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CONTENTS OF THIS ISSUE

The Colorimetric Determination of Certain Local Anesthetics With Sodium 1,2-Naphthoquinone-4-sulfonate 197
Edward G. Feldmann

Catalytic Hydrogenolysis of Halogenated Benzene and Heterocyclic Compounds 201
William O. Foye and *Lewis E. Stoyle, Jr.*

Antipyretic and Toxicity Studies With Acetanilid and *o*-, *m*-, and *p*-Chloroacetanilid 204
M. F. Argus, M. P. Newell, J. T. Henderson, and *F. E. Ray*

The Optical Crystallographic Properties of the Xanthyl Derivatives of Some Barbiturates 207
Edward A. Julian and *Elmer M. Plein*

The Biological Properties of Chlorindanol, A New Antiseptic Agent 212
John Hays Bailey, Frederick Coulston, and *D. A. Berberian*

The Analysis of Digoxin Preparations 217
Albert E. H. Houk, Thomas G. Alexander, and *Daniel Banes*

The Effect of Mouthwashes on the Oral Flora 219
Morris Ostrolenk and *William Weiss*

Paper Chromatographic Evaluation of Menthol 222
Lola V. Hopkins and *Martin I. Blake*

Neurotoxicity of Hydrogen Cyanide 224
Seymour Levine and *Benjamin Weinstein*

A Comprehensive Pharmaceutical Stability Testing Laboratory I. Physical Layout of Laboratory and Facilities Available for Stability Testing 226
Leon Lachman and *Jack Cooper*

A Comprehensive Pharmaceutical Stability Testing Laboratory II. Record and Control System Employed for the Laboratory 233
Leon Lachman and *Jack Cooper*

A Folic Acid Method Involving Permanganate Oxidation 236
S. S. Schiaffino, J. M. Webb, H. W. Loy, and *O. L. Kline*

A Colorimetric Assay for Unoxidized Phenothiazine Derivatives: A New Complex Salt 240
James A. Ryan

Enteric Coatings II. Starch (and Amylose) Acetate Phthalates 244
John G. Wagner, Thomas W. Brignall, and *Stuart Long*

A Chromatographic Technique for the Identification of Fluorescein and Phenolphthalein Derivatives 249
C. Robert McMillion and *H. A. Brown Dunning, Jr.*

Some *in Vitro* Effects of Chlorpromazine, Lysergic Acid Diethylamide, and 5-Hydroxytryptamine on the Respiration of Rat Brain 251
Wesley C. Starbuck and *Harold C. Heim*

Convulsant Properties of Oral Pentylenetetrazol Mixtures 254
Eugene A. Conrad, J. Maxwell Little, and *George H. Armstrong*

A Note on the Alkaloids of *Vinca rosea* Linn. (*Catharanthus roseus* G. Don.) II. Catharanthine, Lochnericine, Vindolinine, and Vindoline 256
Marvin Gorman, Norbert Neuss, Gordon H. Svoboda, Albert J. Banes, Jr., and *Nancy J. Cone*

Book Notices 258

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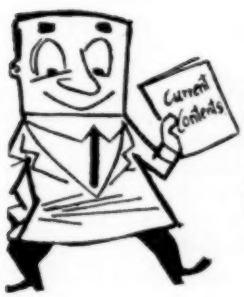

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JOURNAL OF THE AMERICAN PHARMACEUTICAL ASSOCIATION

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The Colorimetric Determination of Certain Local Anesthetics With Sodium 1,2-Naphthoquinone-4-sulfonate*†

By EDWARD G. FELDMANN

The quantitative reaction between sodium 1,2-naphthoquinone-4-sulfonate and primary amines has been applied to the assay of certain local anesthetic compounds. The improved colorimetric method which is employed is particularly useful since it is simple and highly selective, and yet provides a relatively high degree of precision and accuracy. While the presence of secondary amines causes interference with most other chemical methods of assay, neither secondary nor tertiary amines display any adverse effect in the procedure described. Specific application to a commercial dosage form is discussed.

LOCAL ANESTHETICS, because of their basic amino groups, have been conveniently assayed for many years by the use of acid-base titration methods. More recently, instrumental methods and particularly ultraviolet spectrophotometry have provided additional and often improved methods of analysis for these compounds. Instrumental methods are especially useful when the anesthetic agents are found incorporated in various dosage forms. However, certain dosage forms on the commercial market include various other ingredients which seriously complicate these conventional methods of analysis.

Any material displaying acidic or basic properties is potentially capable of interference with an acid-base titration. Often these materials are difficult to extract or otherwise remove for this same reason. Similarly, compounds which display absorption, particularly in the long wavelength regions of the ultraviolet spectrum, will cause interference in simple ultraviolet methods of assay. For these reasons, a more selective method for the determination of certain local anesthetic compounds was sought. The classical Folin method for the determination of amino acids in blood (1) provides a simple, direct, and relatively accurate method for the determination of compounds containing a primary amine group. While this procedure has been modified by several investigators (2–7), it has received little attention as a method for the determination of other primary amino compounds which might be present in other systems. Vonesch and Guagnini (8) have employed one modification of this method for the determination of local anesthetics. The specific procedure employed by these investigators, however, suffers in several respects: (*a*) while the color producing reaction proceeds most quantitatively under slightly alkaline conditions (9), no provision is made to adjust the pH of the reaction; (*b*) bleaching the excess reagent with strong alkali following the reaction gives rise to interfering reaction products; (*c*) since the colored complex produced by many local anesthetics is insoluble in dilute alkali except at very low concentrations, the determination of many local anesthetics is seriously limited; and (*d*) the resulting relationship between con-

* Received September 20, 1958, from the Division of Chemistry, American Dental Association, Chicago, Ill.

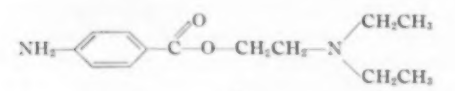
† Analysis of Local Anesthetics II. For the preceding paper in this series see THIS JOURNAL, **47**, 676(1958).

The author wishes to express his appreciation to Dr. J. Roy Doty and Mr. Henry M. Koehler for their many helpful comments, and to Mrs. Helen Jones for her assistance in preparing the manuscript.

centration and color intensity is somewhat nonlinear.

Furman and co-workers (7) have reported a greatly improved modification of the Folin procedure and have employed it with good success in the determination of amino acids. This method, when employed in the determination of local anesthetics, was found to be highly satisfactory in this laboratory. The procedure has none of the disadvantages of the Vonesch and Guagnini method which have been noted above. The Furman modification was found to give a reproducible straight-line relationship between color development and concentration for those local anesthetics containing primary amino groups. Moreover, other anesthetics having secondary or tertiary amino groups, but no primary amines, produced no color, and therefore gave no interference in the procedure. As a result, this method provides a means of assaying selectively for one anesthetic agent containing a primary amine in the presence of a second anesthetic having only secondary and tertiary amino groups.

One such dosage form mixture is currently being marketed in dentistry. This product[1] contains both 2′-diethylaminoethyl 4-aminobenzoate hydrochloride (procaine hydrochloride) and 2′-dimethylaminoethyl 4-butylaminobenzoate hydrochloride (tetracaine hydrochloride). The structural formulas for procaine (I) and for tetracaine (II) are illustrated. Because of the



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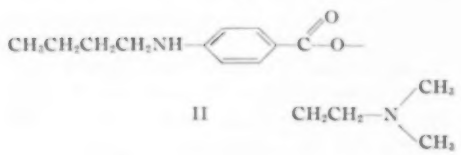

II

very similar chemical and physical properties of these two drugs, they cannot be assayed directly by acid-base titration, nor can they be conveniently quantitatively separated. Moreover, their ultraviolet properties are such that they give rise to mutual absorption interference (see Fig. 1) and as a result, direct determination is difficult and subject to relatively large errors. The assay procedure described herein, however, enables the direct determination of micro quantities of the primary amine procaine, without interference from the presence of the nonprimary amine tetracaine. Pratt (10) recently described a procedure using a nitrous acid titration for the determination of procaine-tetracaine mixtures. His procedure, however, suffers from the need of a large sample, and from the fact that a specially constructed titration chamber is required.

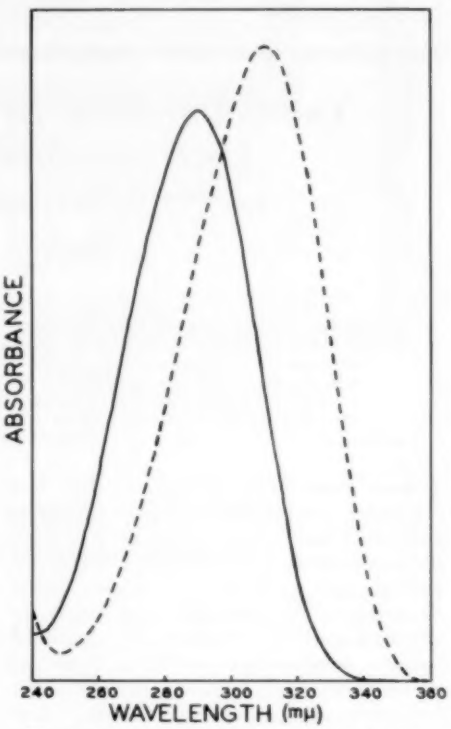

Fig. 1.—Ultraviolet absorbance curves of aqueous solutions; —— procaine hydrochloride; - - - - tetracaine hydrochloride; each 0.01 mg. per ml.

EXPERIMENTAL

Chemicals.—Samples of the local anesthetics employed were generously supplied by their manufacturers: tetracaine hydrochloride and propoxycaine hydrochloride by Cook-Waite Laboratories, Inc., Subsidiary of Sterling Drug; butethamine hydrochloride, metabutethamine hydrochloride, metabutoxycaine hydrochloride, and naepaine hydrochloride by Novocol Chemical Manufacturing Co., Inc.; butacaine sulfate, ethyl aminobenzoate, and procaine hydrochloride by Abbott Laboratories; lidocaine by Astra Pharmaceutical Products, Inc.; meprylcaine hydrochloride by Oradent Chemical Co. Inc.; and piperocaine hydrochloride by Eli Lilly and Co. Sodium 1,2-naphthoquinone-4-sulfonate (Eastman Kodak white label) was employed without fur-

[1] Cook-Waite Laboratories, Inc., markets a solution for injection containing 2.0% procaine hydrochloride (Novocain) and 0.15% tetracaine hydrochloride (Pontocaine Hydrochloride).

ther purification. All other chemicals used were the highest grade of the commercially available materials.

Apparatus.—A Beckman Model DK-2 recording spectrophotometer, with silica cells of 1.00 cm. light path, was employed to record the absorption spectra. Absorbance values at peak wavelengths were checked using a Beckman DU spectrophotometer. All other absorption measurements, including those for the preparation of standard curves, were made employing a Klett-Summerson colorimeter with a No. 50 filter.

Procedure.—A sample of the unknown solution and graded aliquots of a stock standard solution, containing a maximum of 3×10^{-6} mole of primary amine, are transferred to 50-ml. volumetric flasks. Each solution is diluted to approximately 20 ml. with water, and exactly 3 ml. of hydrochloric acid (0.050 *N*) and 3 ml. of sodium carbonate (0.114 *N*) are added. The solutions are mixed, 1 ml. of 1.0% sodium 1,2-naphthoquinone-4-sulfonate solution (prepared fresh each day) is added, the solutions are again mixed, and are allowed to stand in the dark for one and one-half to two hours. Excess reagent is then bleached by the addition of 1 ml. of acetate buffer (25 Gm. sodium acetate trihydrate and 250 ml. glacial acetic acid per liter) and 3 ml. of 4% sodium thiosulfate solution. The solutions are then made up to volume and mixed. Colorimeter readings are taken between ten and twenty minutes from the time that the thiosulfate solution is introduced.

RESULTS AND DISCUSSION

Reaction Involved and Influence of Chemical Structure.—In this assay procedure the yellow sodium 1,2-naphthoquinone-4-sulfonate, in the presence of alkali, reacts with a primary amine-containing compound to yield a highly colored red or orange-red product. Chemically this colored product is an imine resulting from the displacement of the sulfonate radical (7, 9). The excess yellow reagent is then bleached with sodium thiosulfate after making the solution slightly acid with acetate buffer. The absorption curve in the visible spectrum for a typical sample of procaine derivative (after bleaching) is shown in Fig. 2. It will be noted that the absorption maximum occurs at 483 mμ. Reagent blank readings at this wavelength are very low.

In comparing absorption curves of the colored solutions obtained with equimolar concentrations of the various local anesthetics, it was noted that the curves were almost identical for structurally similar local anesthetics. Compounds such as butethamine, propoxycaine, butacaine, and naepaine all produce color which absorbs at the same wavelength maximum and with essentially the same intensity as does procaine. Structurally all of these compounds have an aromatic primary amino group in the position *para* to the carboxylic ester linkage. Metabutethamine and metabutoxycaine, both of which contain an aromatic primary amine in the position *meta* to the ester linkage, show somewhat different absorption characteristics (see Fig. 2). This is in keeping with the findings reported (7) for the amino acids; this difference is attributed to the character of the inductive effect caused by the R group of the amine. It can be seen from the figure that the absorption

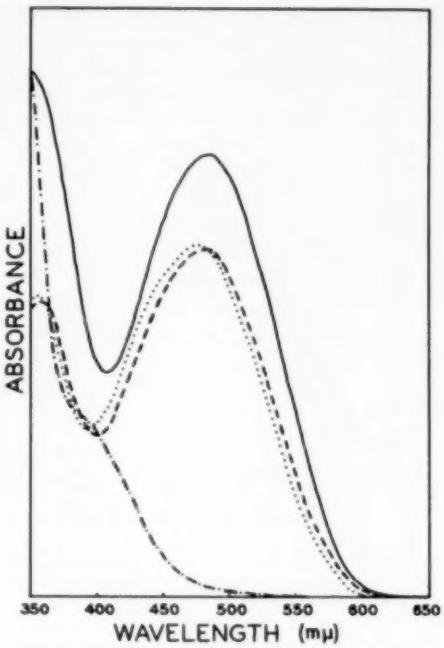

Fig. 2.—Visible absorbance curves of color complexes; —— procaine complex; – – – – metabutethamine complex; . . . . metabutoxycaine complex; —·—·— reagent blank.

curves for these *meta* amino compounds are less intense and the maxima occur at somewhat lower wavelengths (about 478 mμ).

The local anesthetics meprylcaine, tetracaine, piperocaine, and lidocaine, each of which has a secondary or tertiary amine group but no primary amine, give no color formation with this procedure.

For the preparation of standard curves, or for routine assay procedures, the color intensity can be conveniently measured by employing a simple filter colorimeter in the region of 500 mμ. A series of such standard curves is presented in Fig. 3. These curves were prepared by plotting observed colorimeter readings *versus* the volumes taken of equimolar concentrations of the various anesthetics. It should be noted that in all cases the ratio of anesthetic concentration to colorimeter reading follows a straight-line relationship; that is, Beer's law holds for this system. It can also be seen that the differences previously noted for the *meta* amines are again in evidence.

Color Development and Stability.—The assay method is essentially a micro procedure, and a maximum of 3×10^{-6} mole of the local anesthetic being determined can be present in the sample. Hence, a maximum of 0.04 ml. of commercial solution containing 2% procaine hydrochloride can be employed as the sample, while 0.02 ml. is preferable. Since the volume of the sample employed can be as great as 20 ml., solutions can be assayed which contain as little as 1.5×10^{-4} mole per ml.; that is, about 3 μg. of local anesthetic per ml. In the course of this investigation it was found that if quantities of the amine larger than 3×10^{-6} mole were present in

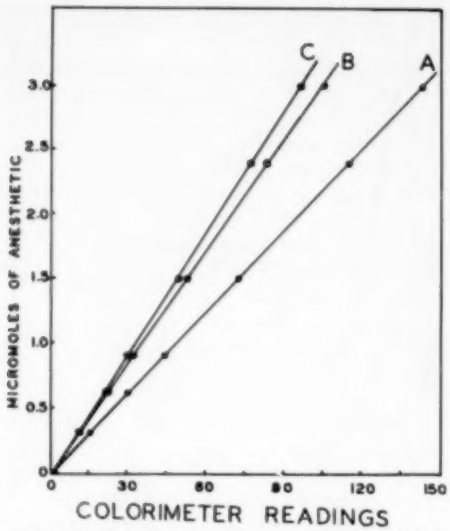

Fig. 3.—Standard curves prepared by plotting anesthetic concentrations *versus* colorimeter readings; A—procaine complex; B—metabutethamine complex; C—metabutoxycaine complex.

the sample, readings which were lower than theoretical resulted. When the concentration of each of the reagents was then doubled, greater color development occurred with the large amine samples, but the colored complex often partially precipitated from solution. By increasing the concentration of only the color developing reagents, and employing normal concentrations of the bleaching reagents, dye precipitation was avoided. However, instrument readings indicated that the color development was no longer as closely proportional to the local anesthetic concentration as had been previously observed when normal concentrations of all of the reagents were employed.

Previous investigators, in discussing the use of sodium 1,2-naphthoquinone-4-sulfonate in the assay of amino acids, have mentioned various time intervals for maximum color development, and little regarding the duration of color stability after bleaching. Folin (1) suggested that the color should be allowed to develop for nineteen to thirty hours; Furman (7) recommended two and one-half hours; and Sahyun (3) suggested ten minutes at 100°. None, however, commented on the stability of the color following bleaching. In this study it was found that results were most reproducible when the color was allowed to develop for one and one half to two hours at room temperature, and the color intensity was determined within ten to twenty minutes from the time that the thiosulfate was introduced. The shorter optimum reaction time here might reflect a greater reactivity of the aromatic amines as compared with the aliphatic amines present in the amino acids. Reading color intensity less than five or ten minutes after addition of the bleaching reagents seems to introduce some slight error due to incomplete bleaching, while readings after twenty minutes are somewhat low due to a gradual fading of the color. Colorimeter readings taken from forty-five to sixty minutes after the addition of thiosulfate were generally about 5% lower than readings taken within twenty minutes. Repeated readings on a series of different samples indicated good color stability for approximately thirty minutes, after which very gradual fading occurs over several hours time.

In the case of several anesthetics, particularly butacaine, naepaine, and ethyl aminobenzoate, it was noted that the solutions were quite turbid after color development, but prior to buffering and bleaching. This was attributed to insolubility of the color complex in alkaline solution, since addition of the acidic acetate buffer, in every case except for ethyl aminobenzoate, served to immediately clarify the solution. Ethyl aminobenzoate, in contrast to the other local anesthetics, does not contain a second amine which is capable of being salted, and therefore solubilization of its complex by acid would not be expected. Only in very dilute concentrations does the ethyl aminobenzoate complex not precipitate, and in these concentrations color formation follows the same curve as that of the other *p*-aminobenzoate anesthetics.

Precision and Accuracy.—Some indication of the precision given by the method can be seen by examination of Table I.

TABLE I.—COLORIMETER READINGS OF REPLICATE PROCAINE HYDROCHLORIDE SAMPLES[a]

Sample	Reading	Sample	Reading
1	70.5	5	69.5
2	70.5	6	70.0
3	69.5	7	70.0
4	69.5	8	69.5
Mean—69.88		Standard Deviation—0.47	

[a] Each sample contained 1.50 μM procaine hydrochloride.

Eight replicates of a procaine hydrochloride standard solution were treated by the method described and the readings obtained on a colorimeter are given in Table I.

A series of procaine hydrochloride-tetracaine hydrochloride mixed solutions was also prepared in which the concentration of procaine was held constant while the tetracaine concentration was varied. The solutions were then assayed for their procaine content. The purpose of this experiment was to ascertain that the presence of tetracaine hydrochloride had no adverse effect on the procaine hydrochloride determination. As seen in Table II, no interference

TABLE II.—ANALYSIS OF KNOWN PROCAINE HYDROCHLORIDE-TETRACAINE HYDROCHLORIDE MIXTURES FOR PROCAINE HYDROCHLORIDE

Sample	Procaine Hydrochloride Actual, μM	Tetracaine Hydrochloride Actual, μM	Procaine Hydrochloride Found, μM	Procaine Hydrochloride, % of Theory
1	1.500	0.000	1.500	100.0
2	1.500	0.060	1.497	99.8
3	1.500	0.150	1.497	99.8
4	1.500	0.300	1.500	100.0
5	1.500	0.600	1.511	100.8
6	1.500	1.500	1.503	100.2
Precision—0.4%				

due to tetracaine was noted—even when the tetracaine hydrochloride was included in over tenfold the concentration present in the commercial mixture.

These data, along with other observations made during the course of this study, indicate that the procedure gives a high degree of precision. Optimum accuracy can be expected in an assay when (*a*) the unknown sample contains from one to two and one-half μM of the local anesthetic being determined, (*b*) standard samples for the preparation of a standard curve are run simultaneously, and (*c*) instrument readings of both unknown and standard samples are taken at the same time interval after addition of the buffer and bleaching reagents. Under these conditions the method should be accurate to ± 1-2%. When a previously prepared standard curve is employed, or instrument readings are taken at different time intervals for the unknown and standard samples, then the error will probably be slightly greater. Even under these conditions an accuracy to ± 3-4% can be expected.

SUMMARY

1. The reaction of sodium 1,2-naphthoquinone-4-sulfonate with a series of local anesthetic compounds has been studied.

2. In the case of anesthetics containing a primary amine the reaction quantitatively yielded a highly colored product, the intensity of which was conveniently measured.

3. Secondary and tertiary amines have been shown not to interfere in the assay.

4. Particular application to a commercial anesthetic mixture has been described.

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Catalytic Hydrogenolysis of Halogenated Benzene and Heterocyclic Compounds*

By WILLIAM O. FOYE and LEWIS E. STOYLE, Jr.†

A relatively general procedure for the catalytic hydrogenolysis of aromatically-bound halogen has been found with the use of 10 per cent palladium-on-charcoal. Benzene and pyridine rings remained unaffected during this reaction, but several other heterocyclic rings were reduced or cleaved.

THE REMOVAL of organically-bound halogen is generally anticipated during catalytic hydrogenation of organic compounds, but hydrogenolytic cleavage as a selective method of removing halogen from organic molecules has not been widely investigated. Busch and Stöve (1) showed that the halogen in a number of aliphatic and aromatic halides was quantitatively removed by hydrogenation over a palladium-on-calcium carbonate catalyst at atmospheric pressure, but in most cases, the nature of the organic residue was not determined. Using the Adams platinum oxide catalyst, Marvel, *et al.* (2), found that the ring was also hydrogenated under conditions where the halogen was reduced from aromatic halides. More recently, Baltzly and Phillips (3) found that in acid or neutral media, aromatically-bound chlorine could be removed by hydrogenation over a laboratory-prepared palladized charcoal catalyst (4) where the halogen was activated by other substituents. Otherwise, aromatically-bound chlorine was stable under conditions that did not reduce the ring, while bromine similarly bound was generally removed at a moderate rate. Raney nickel has also been used for the quantitative removal of organically-bound halogen (5).

Since a catalyst has been found which is capable of removing chlorine from the s-triazine ring without reduction of the ring (6), it was decided to investigate its use for the removal of other aromatically-bound halogen. The catalyst is 10 per cent palladium-on-charcoal prepared by the American Platinum Works (Newark, N. J.), and it was found, in general, to remove halogen bound to aromatic rings using a pressure of 2–3 atmospheres of hydrogen at room temperature or slightly higher (no attempt was made to hold the temperature constant during the reduction). No attempts to regulate the acidity or basicity

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of the media were made, and no extensive purifications were necessary for the starting materials.

In aromatic halides containing the benzene ring, the ring remained unreduced during removal of chlorine or bromine as expected for palladium catalysts. Although Baltzly and Phillips (3) found that the chlorophenols and chlorobenzoic acids, as well as *p*-bromobenzoic acid, resisted reduction with a palladized charcoal catalyst under conditions where the ring was unreduced, by our procedure both chloro- and bromobenzoic acids and *p*-bromophenol were readily dehalogenated. *p*-Bromobenzoic acid, in fact, was rapidly hydrogenolyzed to benzoic acid with a 78 per cent yield.

Among the heterocyclic halides that were treated by this procedure, only the pyridine ring remained unreduced using 2-chloropyridine. 2-Chloroquinoline was also dehalogenated but partially reduced to 1,2,3,4-tetrahydroquinoline. 2-Chlorobenzoxazole was likewise dehalogenated, but the heterocyclic ring was cleaved leaving aniline hydrochloride as the reduction product. The catalyst was poisoned by sulfur-containing heterocyclic compounds, since addition of fresh catalyst to the inhibited reactions caused further uptake of hydrogen. No identified products were isolated from these reactions, carried out on 2-chlorobenzothiazole and 2-chlorothiophene.

Nitro groups were also readily reduced to amino groups in the presence of halogen, carboxyl, carboxamide, and sulfonamide substituents. Reduction of *p*-bromophenacyl bromide removed only the aromatically-bound bromine to give phenacyl bromide. This result is not surprising, since Baltzly and Phillips (3) found that aliphatically-bound halogen was more resistant to hydrogenolysis than aromatically-bound halogen. With Adams platinum oxide catalyst, both the ring and ketone group of acetophenone are reduced (2). 4,4′-Dibromobiphenyl was the only aromatic halide used that resisted hydrogenolysis by this method.

The compounds hydrogenolyzed with 10 per cent palladium-on-charcoal and their products are recorded in Table I. The times required for removal of halogen are also included, and it can be seen that *para*-substituted electropositive substituents facilitate the removal. This result agrees with the findings of Baltzly and Phillips (3) where amino substituents were shown to be activating. In this connection, it is apparent that nitro groups are reduced to amino groups prior to the removal of halogen, since *p*-chloroaniline and *p*-chloronitrobenzene are reduced in correspondingly similar lengths of time. From a comparison of the times of reaction of the first eight compounds listed in Table I, where the same reaction conditions (molar quantity, concentration, temperature, pressure) prevailed, it may be concluded that in the neutral catalytic hydrogenolysis of aromatic halogen, *para*-substituents affect the ease of halogen removal, and electropositive substituents favor the removal.

EXPERIMENTAL

Materials.—Most of the compounds used were Eastman Organic Chemicals of the best grade available. No purifications were carried out. The remainder were synthesized as follows.

4-Chloro-3-nitrobenzamide.—This compound was prepared from 3-nitro-4-chlorobenzoic acid by means of thionyl chloride and aqueous ammonia. The product was recrystallized from ethanol to give a 75% yield of amide, m. p. 151–153°. Montagne (7) lists a m. p. of 156°.

5-Nitro-2-thiophenecarboxylic Acid.—This compound was prepared by the procedure of Dann (8). The product melted at 155–156°, which agrees with the reported value.

4,4′-Dibromobiphenyl.—This compound was prepared according to the method of Scholl and Neovius (9). An 80% yield of product was obtained after recrystallization from benzene which melted at 164.5–165.5°, which agrees with the reported value.

***p*-Nitrobenzenesulfonamide.**—This was prepared by heating *p*-nitrobenzenesulfonyl chloride for two hours with aqueous ammonia and sodium hydroxide. A 78% yield of the amide was obtained, m. p. 179–180°, which agrees with the reported value (10).

Method.—The apparatus used was a standard Catalytic Hydrogenation Apparatus, Low Pressure, Shaker Type made by the Parr Instrument Co. The catalyst employed was 10% palladium-on-charcoal manufactured by the American Platinum Works, Newark, N. J. The only preparation required before using this catalyst was oven-drying at 60°.

Reductions were carried out using 0.5 Gm. of catalyst per 0.01 mole of organic compound, with a minimum of 0.5 Gm. for any reaction. The pressure of hydrogen used varied from 2–3 atmospheres. The quantity of the substrate varied according to availability and solubility, but was generally from 0.01–0.05 mole. The amount of solvent employed was either 50 or 75 ml., and no purifications were carried out; the best grade available was generally used. The temperature of the reaction was not controlled and was taken as that of the laboratory except in some reductions, particularly where nitro groups were present, where temperature rises were noted. Reduction was considered complete when the hydrogen pressure remained constant for thirty minutes. The time of reduction was taken up to the point where constancy of pressure was first reached, however.

An attempt to maintain constancy of reaction conditions was made for the first eight compounds in Table I. In these cases, 0.01 mole of compound in 50 ml. of solvent with 0.5 Gm. of catalyst was shaken with approximately 2 atmospheres of hydrogen with the starting temperature at approximately 24°.

TABLE I.—HYDROGENOLYSES WITH 10% PALLADIUM-ON-CHARCOAL

Substrate	Moles	Solvent	Temp., °C.	Time, min.	Product	M.p., °C.	Lit. M.p., °C.	Yield, %
p-Chloroaniline	0.01	Methanol	25	3	Aniline·HCl	197–198	198	90
p-Bromoaniline	0.01	Methanol	25	2	Aniline·HBr	150 (s); 275 (m)	286	64
p-Chlorobenzoic acid	0.01	Ethanol	27	5	Benzoic acid	121–122	122	65
p-Bromobenzoic acid	0.01	Abs. ethanol	33	7	Benzoic acid	121–122	122	78
2,4′-Dibromoacetophenone	0.01	Ethyl acetate	25	8	Phenacyl bromide	49–50	50	67
p-Bromophenol	0.01	Methanol	26	2	Phenol	.....	...	...[a]
1-Chloro-4-nitrobenzene	0.01	Methanol	31	5	Aniline·HCl	197–198	198	46
1-Chloro-2,4-dinitrobenzene	0.01	Methanol	31	10	*m*-Phenylene-diamine·HCl	277–278	...[b]	89
4-Chloro-3-nitrobenzamide	0.005	Methanol	26	9	*m*-Aminobenz-amide·HCl	238–240	...[c]	...
p-Nitrobenzenesulfonamide	0.01	Methanol	29	12	Sulfanilamide	165–166	165–166	93
p-Nitrobenzoic acid	0.02	Methanol	28	4	*p*-Aminobenzoic acid	186–187	187–188	89
4,4′-Dibromobiphenyl	0.01	Benzene	26	5	4,4′-Dibromobi-phenyl	163–164	164–165	...
2-Chloropyridine	0.05	Methanol	29	30	Pyridine·HCl	80–82	82	80
2-Chloroquinoline	0.025	Methanol	29	135	1,2,3,4-Tetrahydro-quinoline·HCl	179–180	180–181	91
2-Chlorobenzoxazole	0.025	Methanol	30	195	Aniline·HCl	197–198	198	94
2-Chlorobenzothiazole	0.05	Ethyl acetate	27	180	.....	.....	...	...
2-Chlorothiophene	0.05	Methanol	31	180	.....	.....	...	...
5-Nitro-2-thiophenecarboxylic acid	0.01	Benzene	29	30	.....	.....	...	...

[a] After removal of solvent, a small amount of oily product was isolated which gave a positive phenol test and a negative halogen test.
[b] No literature m. p. could be found for this derivative, so it was converted to the free base, m. p. 61–62°.
[c] No literature m. p. could be found for this derivative, so it was converted to the free base, m. p. 78–79°.

The reduction products were isolated after filtration of the catalyst by evaporation of the solvent. They were then recrystallized to constant melting point. The melting points were taken on a Fisher-Johns block and are uncorrected. Qualitative tests for ionizable halogen were carried out with all the products.

SUMMARY

1. Catalytic hydrogenolysis of aromatically-bound halogen was found to be relatively general using a commercially-prepared 10 per cent palladium-on-charcoal catalyst. Several aryl halides not previously amenable to reduction by this method were readily hydrogenolyzed. The only example noted where the halogen was resistant to reduction was 4,4′-dibromobiphenyl.

2. Nitro groups were also readily reduced to amino groups by this procedure in the presence and absence of halogen removal.

3. Several heterocyclic rings were found to be reduced with this procedure, but the pyridine, as well as the benzene, ring remained unaffected.

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Antipyretic and Toxicity Studies With Acetanilid and *o*-, *m*-, and *p*-Chloroacetanilid*

By M. F. ARGUS, M. P. NEWELL, J. T. HENDERSON, and F. E. RAY

The LD_{50} of *o*-, *m*-, and *p*-chloroacetanilid and of acetanilid was determined in mice and rats. The decreasing order of relative toxicity was the same in both species: *p*-chloroacetanilid, *m*-chloroacetanilid, acetanilid, and *o*-chloroacetanilid. The antipyretic activity of the chloroisomers in the rat decreases in the same sequence as their toxicity.

RING SUBSTITUTION of the antipyretic drug, acetanilid, has an important influence on the metabolic behavior of this compound. Thus, Bray, *et al.* (1), have found that the extent of deacetylation depends on the position of substituents in this molecule. Later, Argus, *et al.* (2), observed that the three ring-substituted monochloroacetanilids display different tissue distribution and urinary excretion patterns following administration to both rats and guinea pigs, suggesting that substitution affects the position and extent of hydroxylation of acetanilid. These alterations in the metabolism of the compound are reflected in changes in the physiological properties of the drug, since with the same dose of *p*-chloroacetanilid and *o*-chloroacetanilid in rats, toxic symptoms were observed only for the former compound.

In the present study a systematic determination of the relative toxicities of *o*-, *m*-, and *p*-chloroacetanilid and unsubstituted acetanilid was made in mice and rats. The effect of the position of ring substitution on antipyretic activity was also investigated.

EXPERIMENTAL

Compounds Used.—The three chloroacetanilids were obtained from the Eastman Chemical Company and were recrystallized from 50% ethanol. Melting points were *o*-chloroacetanilid, 88°; *m*-chloroacetanilid, 72°; and *p*-chloroacetanilid, 177°. Merck acetanilid was recrystallized from water to a melting point of 114°.

Toxicity Experiments.—Female C57bl/6 mice (18 weeks; 20 Gm. ± 2 Gm.) and Sprague-Dawley rats (210–260 Gm.) were employed. Rats were housed 2 to a cage and mice 10 to a cage in an air-conditioned room maintained at 22°. The animals had access to Purina Laboratory Chow and water at all times.

Preliminary tests to determine the dosage range for the toxicity study were made using 2 mice per dose. When the approximate levels were established, groups of 10 mice and 4 rats for each dose level of the isomeric chloroacetanilids and acetanilid were used. Once the LD_{50} values were found for the groups of mice, smaller groups of rats were employed to determine whether the order of toxicity of the compounds was the same in both species. The drugs were administered on a mg./Kg. of body weight basis. Each dose was suspended in 0.6 cc. of 2% Methocel and injected intraperitoneally. The dosages tested are given in Table I. Percentage mortality was based on deaths occurring within seventy-two hours after administration of the drug.

The relative acute toxicities of acetanilid and *o*-, *m*-, and *p*-chloroacetanilid were determined statistically in terms of LD_{50} after Burn, *et al.* (3). In this method the percentage of mortality is plotted against the dosage administered on logarithmic probit paper. The dosage level, at the point where the curve crosses probit 5 on the graph, is taken as the LD_{50}.

Antipyretic Experiments.—Female Sprague-Dawley rats (130–250 Gm.) were employed in groups of 12 animals. Rats were housed two to a cage and all experiments were carried out in a room maintained at 22°. The drugs were given by stomach tube at a concentration of 0.125% suspended in 2% gum acacia. The dosage was 12.5 mg./Kg. body weight. Sixteen hours prior to administration of each compound, fever was induced in the rats by the injection of 15% brewers' yeast (Fisher No. Y-1) suspended in 35% acacia. A subcutaneous injection of 1 cc./100 Gm. body weight produced a 1.5 to 2° rise in the rectal temperature at twelve hours, and this fever then persisted for about eighteen hours. Food (Purina Laboratory Chow) was removed from the animal cages four hours prior to injection of the yeast and twenty hours before administration of the drugs. The animals had free access to water at all times. Groups of eight fevered controls and six nonfevered controls were run simultaneously with each drug-test group.

Rectal temperature determinations were made using a Thermistor Probe (Sargent plain, low range, thermometric element No. S-81620). This probe was connected through a Thermistor Bridge to a Brown Potentiometer and Recorder [Model No. Y153X12V-X-30AKN4(V)]. By inserting the probe in tap water at 34° and 42°, the potentiometer and recorder were standardized, through adjusting the controls on the Thermistor Bridge, so that a reading of "0" on the instrument scale represented 34° and "28" on the instrument represented 42°. A graph paper scale was then superimposed on the instrument scale so that readings to 0.1° could be made. The entire tip of the probe was inserted into the rectum of the rat; five seconds was sufficient for accurate temperature readings.

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RESULTS AND DISCUSSION

Toxicity Experiments.—Table I has been included to give the complete range of toxicities in C57bl/6 mice and in Sprague-Dawley rats, including the doses corresponding to 0 and 100% mortalities. These last two values, however, are not used (3) in the probit coordinate system (Figs. 1 and 2).

The LD_{50} values obtained for C57bl/6 mice with the probit coordinate system (Fig. 1) are given in Table II as mg./Kg. and mM/Kg. The LD_{50} of *p*-chloroacetanilid is much lower than that of *m*- and *o*-chloroacetanilid or acetanilid. On a mg./Kg. basis, the LD_{50} of *o*-chloroacetanilid is greater than that of acetanilid. Yet, on a mM/Kg. basis, the LD_{50} of the two drugs is the same. These figures indicate that the introduction of the chlorosubstituent, *per se*, does not increase the toxicity of the compound. It is the position of the substituent which seems to be of importance.

TABLE I.—TOXICITY OF *o*-, *m*-, AND *p*-CHLOROACETANILID AND ACETANILID FOLLOWING INTRAPERITONEAL INJECTION TO C57bl/6 MICE AND SPRAGUE-DAWLEY RATS

Compound	C57bl/6 Mice: Dosage,[a] mg./Kg.	C57bl/6 Mice: Mortality, %	Sprague-Dawley Rats: Dosage,[b] mg./Kg.	Sprague-Dawley Rats: Mortality, %
p-Chloroacetanilid	182	0	237	25
	202	10	246	50
	220	20	255	75
	228	70	278[c]	100
	284	100	284[c]	100
m-Chloroacetanilid	500	10	278	0
	589	50	302	50
	700	70	360	100
	980	100	425	75
	...	...	500[c]	100
o-Chloroacetanilid	700	0	700	25
	830	40	760	75
	980	50	830	50
	1370	90	980	75
	...	...	1370	100
Acetanilid	500	10	425	0
	700	30	500	25
	760	50	589	75
	830	90	700	100
	980	90	...	...

[a] Ten animals injected at each dosage level.
[b] Four animals injected at each dosage level except as noted.
[c] Two animals injected.

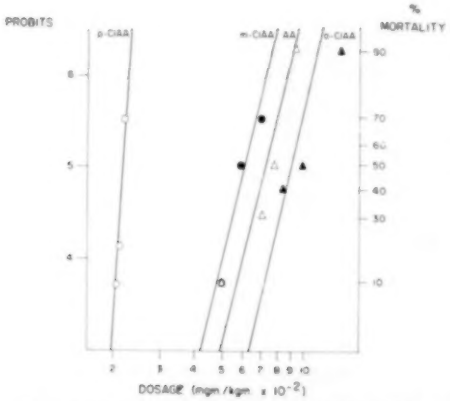

Fig. 1.—Dosage of *p*-chloroacetanilid (*p*-ClAA), *m*-chloroacetanilid (*m*-ClAA), acetanilid (AA), and *o*-chloroacetanilid (*o*-ClAA), plotted against percentage mortality in probit units. The drugs were administered intraperitoneally to C57bl/6 mice.

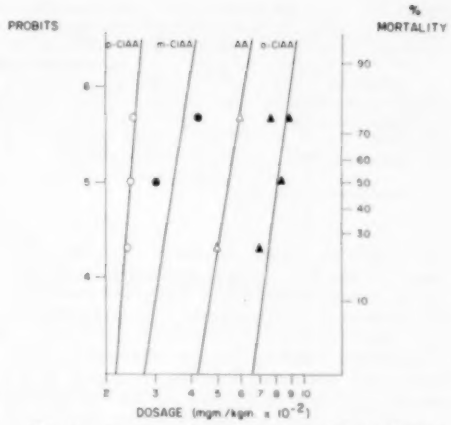

Fig. 2.—Dosage of *p*-chloroacetanilid (*p*-ClAA), *m*-chloroacetanilid (*m*-ClAA), acetanilid (AA), and *o*-chloroacetanilid (*o*-ClAA) plotted against percentage mortality in probit units. The drugs were administered intraperitoneally to Sprague-Dawley rats.

For Sprague-Dawley rats the LD_{50} values (Fig. 2 and Table II) show that the relative toxicities of the isomeric chloroacetanilids and acetanilid are the same for the rat as for the mouse. *p*-Chloroacetanilid is the most toxic drug, followed in order by *m*-chloroacetanilid, acetanilid, and *o*-chloroacetanilid. In rats *o*-chloroacetanilid is less toxic than acetanilid whether the LD_{50} is expressed on a mg./Kg. or a mM/Kg. basis.

In both species *p*-chloroacetanilid has the narrowest dosage range between 0 and 100% mortality. This compound appears to be more toxic to the mice used in these experiments than to the rats. In a previous study on the toxicity of orally administered acetanilid in young albino rats, Smith and Hambourger reported an LD_{50} of 800 mg./Kg. (4). This is somewhat higher than our value for C57bl/6 mice (715 mg./Kg.) and considerably higher than our findings for Sprague-Dawley rats (540 mg./Kg.). In the present investigation, however, acetanilid was administered by the intraperitoneal route, and the differences found for the rat in these two studies are probably due to the difference in the rate of absorption from the gastrointestinal tract and from the peritoneal cavity.

In our studies on the excretion pattern of *o*-, *m*-, and *p*-chloroacetanilid we found (2) that the time required for peak excretion of the *p*-compound is four times that needed for the *o*- and *m*-derivatives in the rat. It is well known that in rodents, acetanilid is detoxicated mainly by conversion to the more soluble *p*-hydroxyaniline (5). Thus, it is not surprising that *p*-chloroacetanilid, in which the *p*-position is blocked, reaches a peak excretion more

TABLE II.—LD_{50} OF *o*-, *m*-, AND *p*-CHLOROACETANILID AND ACETANILID IN C57bl/6 MICE AND SPRAGUE-DAWLEY RATS FOLLOWING INTRAPERITONEAL ADMINISTRATION

Compound	LD_{50} in C57bl/6 Mice[a] mg./Kg.	mM/Kg.	LD_{50} in Sprague-Dawley Rats[a] mg./Kg.	mM/Kg.
p-Chloroacetanilid	225 ± 4.35	1.3 ± .025	245 ± 5.07	1.4 ± .029
m-Chloroacetanilid	610 ± 26.77	3.6 ± .158	350 ± 55.18	2.1 ± .331
Acetanilid	715 ± 31.59	5.3 ± .234	540 ± 28.84	4.0 ± .214
o-Chloroacetanilid	900 ± 74.42	5.3 ± .438	810 ± 53.57	4.7 ± .311

[a] Including standard error.

slowly than the *o*- and *m*-isomers. This longer retention of the *p*-substituted compound by the animal body is now reflected in the greater toxicity of this compound when compared to *o*- and *m*-chloroacetanilid.

Antipyretic Studies.—In the antipyretic experiments with acetanilid, the first depression in the rectal temperature of Sprague-Dawley rats is noted one-half hour after oral administration of the drug in a dosage of 12.5 mg./Kg. (Fig. 3). The greatest depression occurs at three hours with a lowering of 1.1°. The effect begins to diminish at five hours, and at nine hours the depression is only 0.2°. Previously, Smith and Hambourger observed a maximum fall in temperature of only 0.6° when the same dose of acetanilid was given to albino rats (4). In their experiments this maximum temperature decrease occurred between sixty and ninety minutes after administration.

The results obtained with the three isomeric chloroacetanilids are given in Fig. 4. *p*-Chloroacetanilid also brings about a lowering of temperature, beginning 0.5 hour after administration. The greatest depression occurs between one and two and one-half hours and is approximately 0.9°, slightly less than the depression for acetanilid in our experiments. At seven hours the antipyretic effect ceases for the *p*-chloro-derivative. The maximum depression caused by *m*-chloroacetanilid is also 0.9°, which occurs at two and one-half hours after administration. As for the *p*-isomer, no effect is apparent after seven hours. *o*-Chloroacetanilid is the least effective of the four compounds tested. No substantial reduction in temperature is noted until two and one-half hours, and it is only 0.6°. Again no depression of temperature exists after seven hours.

In conclusion, all three isomeric chloroacetanilids have a lower antipyretic activity than acetanilid. In the group of the chloro-isomers the antipyretic activity decreases in the same order as toxicity. There does not seem to be, however, a general correlation between these two pharmacological properties for all four compounds, since *p*- and *m*-chloroacetanilid are more toxic than acetanilid.

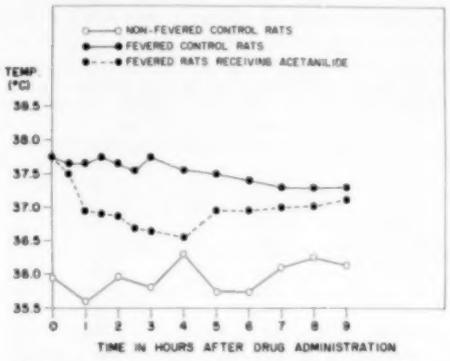

Fig. 3.—Each point on the broken line curve represents the average rectal temperature of 12 Sprague-Dawley rats receiving 12.5 mg. acetanilid/Kg. body weight. The average values for eight fevered control rats and six nonfevered control rats are also plotted.

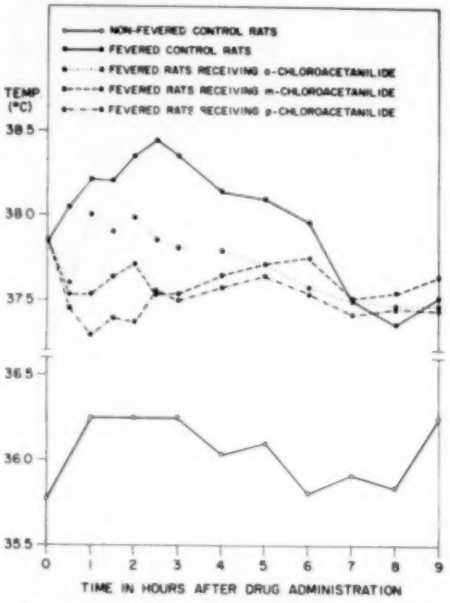

Fig. 4.—Each point on the drug-test graphs represents the average rectal temperature of 12 Sprague-Dawley rats receiving 12.5 mg. *p*-, *m*-, or *o*-chloroacetanilid/Kg. body weight. The average values for eight fevered control rats and six nonfevered control rats are also plotted.

SUMMARY

1. The toxicity expressed as LD_{50}, and the antipyretic activity of acetanilid and of *o*-, *m*-, and *p*-chloroacetanilid were determined in C57bl/6 mice and Sprague-Dawley rats.

2. The relative toxicities on a mg./Kg. basis decrease in both species in the following order: *p*-chloroacetanilid, *m*-chloroacetanilid, acetanilid, and *o*-chloroacetanilid. It has been suggested in

connection with previous studies (2) that the high toxicity of the *p*-chloro-isomer may be due to the blocking of *p*-hydroxylation.

3. The order of the antipyretic activity in rats of these four compounds is the following: acetanilid > *p*-chloroacetanilid > *m*-chloroacetanilid > *o*-chloroacetanilid.

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The Optical Crystallographic Properties of the Xanthyl Derivatives of Some Barbiturates*

By EDWARD A. JULIAN† and ELMER M. PLEIN

The optical crystallographic properties of the xanthyl derivatives of fourteen commonly-used barbiturates and the micro-melting points of these same derivatives and five additional ones are presented. The crystallographic properties, especially the three principal indexes of refraction, readily serve to identify the compounds in question. The crystallographic properties are of particular value when the melting points of the derivatives are close in range.

ONE OF THE PHYSICAL METHODS suggested for the qualitative analysis of the barbiturates is optical crystallography employing the petrographic microscope. The barbiturates, *per se* (1), and benzyl and phenacyl derivatives of barbiturates (2–5) have been studied by this method.

Xanthydrol has been used as a reagent for the identification of barbiturates with melting points as the criteria (6–10). This study presents optical crystallographic data of xanthyl derivatives of barbiturates which can be used as a means of identification.

EXPERIMENTAL

The method followed to obtain xanthyl derivatives of the barbiturates was that of McCutcheon and Plein (10). One gram of xanthydrol (Eastman Kodak Company) was dissolved in 10 ml. of glacial acetic acid to which was then added 0.6 Gm. of barbiturate. The mixture was heated on a water bath for thirty minutes. With some derivatives the compound formed immediately, whereas others did not appear until the next day. The product was washed free of acid and xanthydrol with 20 ml. of ethanol and then dissolved with the heat of a water bath in a minimum quantity of *n*-amyl acetate and acetone (1:1). The solution was filtered into an Erlenmeyer flask and crystallization allowed to proceed slowly. In the majority of cases crystals separated out within a few hours.

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Presented to the Scientific Section, A. PH. A., Detroit Meeting, April, 1956.
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The compounds were air-dried and checked for purity by using melting points listed in the literature as criteria (except for Butisol, see Table I) (6–10). Melting points were obtained with the Kofler micro-melting-point hot stage.

The critical optical crystallographic properties were determined by methods described by Chamot and Mason (11) and Winchell (12).

Diagnostic properties of crystals in their most frequently occurring orientations were determined by procedures previously described (13).

RESULTS AND DISCUSSION

In Table I are listed the micro-melting points for the various xanthyl derivatives of barbiturates identified by trade names, manufacturers', and generic or chemical names. The reagent, xanthydrol, and xanthone, an oxidation product, are included in the table also.

The optical properties of xanthyl derivatives of some barbiturates, xanthydrol, and xanthone are listed in Table II. The highest index of refraction in the authors' set of liquids was 1.785; hence no value above this figure is reported. It was necessary to use crushed specimens in order to obtain some refractive indexes.

Many of the xanthyl derivatives are so flattened that they tend to assume a common orientation on the microscope slide. In Table III are recorded some optical and crystallographic properties observed on the most frequently occurring orientation of the crystals. The optical orientation designated as acute, obtuse, or optic normal indicates that a centered interference figure is found when the crystal, in its most frequently occurring orientation is examined under conoscopic vision. The descriptive term, "inclined," indicates that the interference figure is not centered. It follows that with an inclined orientation one or both of the refractive indexes for the front view of the crystal cannot be determined within narrow limits and are therefore listed as vari-

TABLE I.—MICRO-MELTING POINTS OF XANTHYL DERIVATIVES

Trade Name	Manufacturer[a]	Generic or Chemical Name	Micro-Melting Point[b] of Xanthyl Derivatives, °C.
Alurate	Roche Labs. Div., Hoffmann-La Roche, Inc.	Aprobarbital	230–231
Amytal	Eli Lilly & Co.	Amobarbital	253–254
Butisol[c]	McNeil Labs. Inc.	Butabarbital	229–230
Cyclopal	The Upjohn Co.	Cyclopentenylallyl-barbituric Acid	235
Delvinal	Merck, Sharp & Dohme	Vinbarbital	227–228
Dial	Ciba Pharmaceutical Products, Inc.	Diallylbarbituric Acid	246
Ipral	E. R. Squibb & Sons	Probarbital	212–214
Luminal	Winthrop Labs., Inc.	Phenobarbital	220–221
Nembutal	Abbott Labs.	Pentobarbital	223
Neonal	Abbott Labs.	Butethal	253–254
Nostal	Ames Co., Inc.	Propallylonal	255–256[d]
Ortal	Parke, Davis & Co.	Hexethal	208
Pentothal	Abbott Labs.	Thiopental	167–168
Pernoston	Ames Co., Inc.	Butallylonal	251[d]
Phanodorn	Winthrop Labs., Inc.	Cyclobarbital	258[d]
Sandoptal	Sandoz Pharmaceuticals, Div. Sandoz, Inc.	Allylbarbituric Acid	248
Seconal	Eli Lilly & Co.	Secobarbital	183
Sigmodal	Ames Co., Inc.	Amylbromallylbar-bituric Acid	199–200
Veronal	Winthrop Labs., Inc.	Barbital	246–248
	Eastman Kodak Co.	Xanthone	176–177
	Eastman Kodak Co.	Xanthydrol	122–123

[a] The authors wish to express their appreciation and thanks to the manufacturers who supplied the barbiturates used in this study.
[b] The "Kofler" micro-melting point hot stage (30–350°) (A. H. Thomas & Co., Philadelphia, Pa.) was used.
[c] Butisol (xanthyl derivative). Calculated for $C_{24}H_{22}O_3N_2$: *N*, 4.89%. Found: *N*, 4.73% by the micro-Dumas method.
[d] Decomposed.

TABLE II.—OPTICAL PROPERTIES OF XANTHYL DERIVATIVES OF SOME BARBITURATES

Xanthyl Derivative	Crystal System[a]	Extinction Angle, Degrees	Optic Sign	Refractive Indexes			Elongation	Dispersion
				Alpha	Beta	Gamma		
Alurate	M	13	+	1.570	1.645	1.725	+	$v > \rho$
Amytal	O	0	−	1.520	1.664	1.730	+	$v > \rho$
Butisol	M	36	−	1.565	1.650	1.716	+	$v > \rho$
Cyclopal	M	36	+	1.580	1.655	1.740	+	$\rho > v$
Dial	M	12	+	1.555	1.645	1.740	+	$\rho > v$
Ipral	T	43	+	1.593	1.617	1.700	±	$v > \rho$
Luminal	M	32	−	1.650	1.696	1.706	±	Crossed
Neonal	M	33	−	1.560	1.645	1.720	−	$v > \rho$
Nostal	M	15	−	1.595	1.685	1.740	+	$v > \rho$
Pentothal	M	21	−	1.608	1.690	1.700	±	$v > \rho$
Pernoston	M	15	−	1.592	1.680	1.730	+	$v > \rho$
Sandoptal	M	8	−	1.565	1.650	1.730	+	$v > \rho$
Sigmodal	M	38	−	1.555	1.675	1.728	+	$v > \rho$
Veronal	M	21	+	1.594	1.620	1.705	+	$v > \rho$
Xanthone	M	28	−	1.680	1.745	1.785	−	$\rho > v$
Xanthydrol	M	27	+	1.590	1.677	>1.785	±	$\rho > v$

[a] M—Monoclinic, D—Orthorhombic, T—Triclinic.

able. Extinction angles are measured from the long axis on the front face of each crystal.

Figures 1 and 2 are orthographic projection drawings of the crystals showing front, side, and top views. These drawings supplement the data presented in Tables II and III and facilitate identification of the barbiturates. To determine the appearance of the crystals for side and top views the crystals were observed and rolled in Canada balsam. The front view of the crystals is generally the usual orientation. Xanthyl derivatives of Amytal and Neonal, however, have ridges down the center of the crystals which make it unlikely that they will be oriented in the position shown. However, this position is presented to illustrate the location of the crystal axes. Dashed lines indicate the vibration directions and refractive indexes are recorded for crystals which show consistent values in these directions. An asterisk indicates the higher value on views where consistent refractive indexes could not be obtained. Crystal angles measured microscopically are shown in the corners of the diagrams.

Additional descriptions of the reagent and derivatives will facilitate identification of the compounds

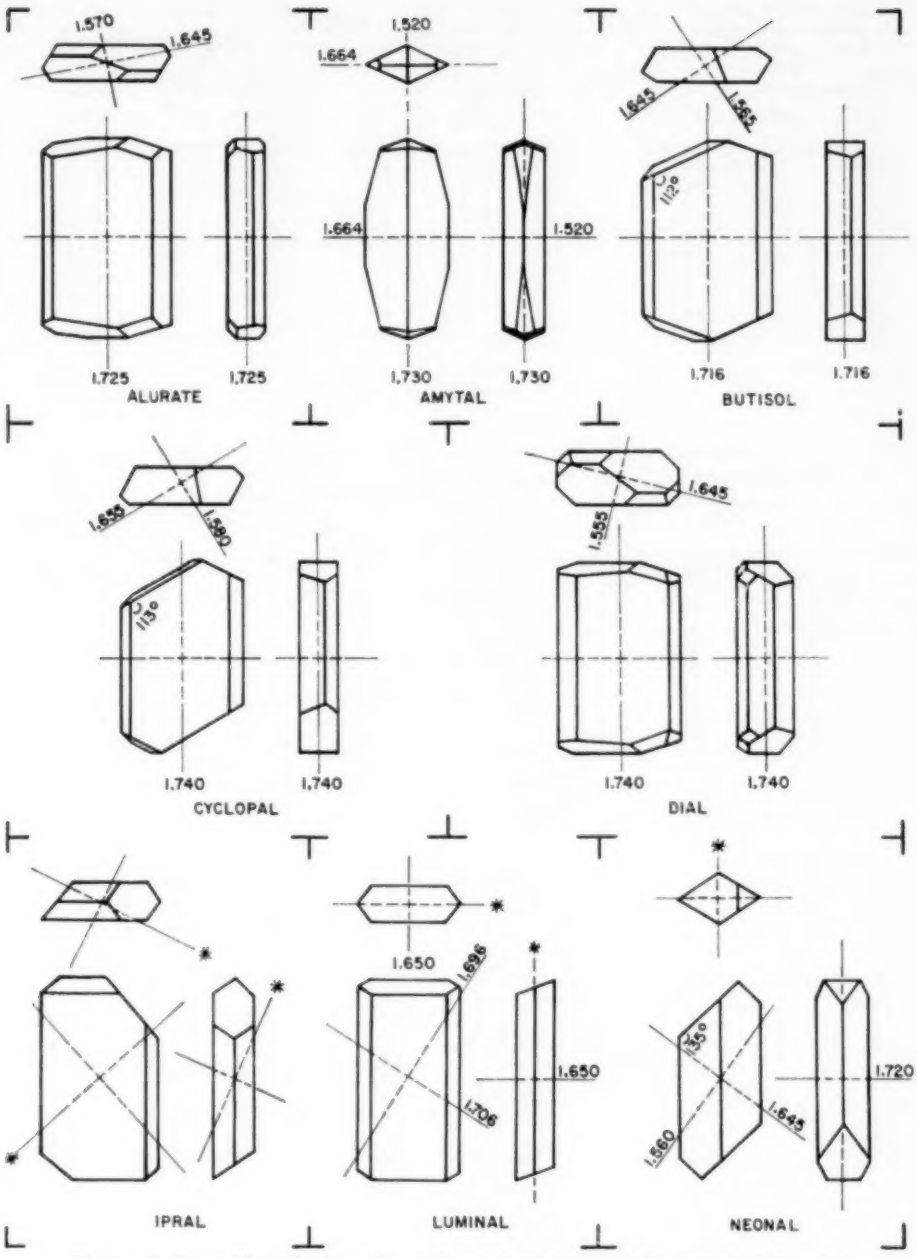

Fig. 1.—Orthographic projections of typical crystals of xanthyl derivatives of barbiturates.

by optical crystallographic methods. The reagent, xanthydrol, is twinned and the variable index across the front face in the usual orientation will be either 1.677 or greater than 1.785. Very few top views of the crystal were found because the crystals are very long and become broken in sampling.

Since xanthydrol is reported to decompose to form xanthone (14), the latter compound was also studied for its optical crystallographic properties. In the course of this investigation, however, no detectable decomposition of xanthydrol occurred. Xanthone formed very long, acicular and taper-

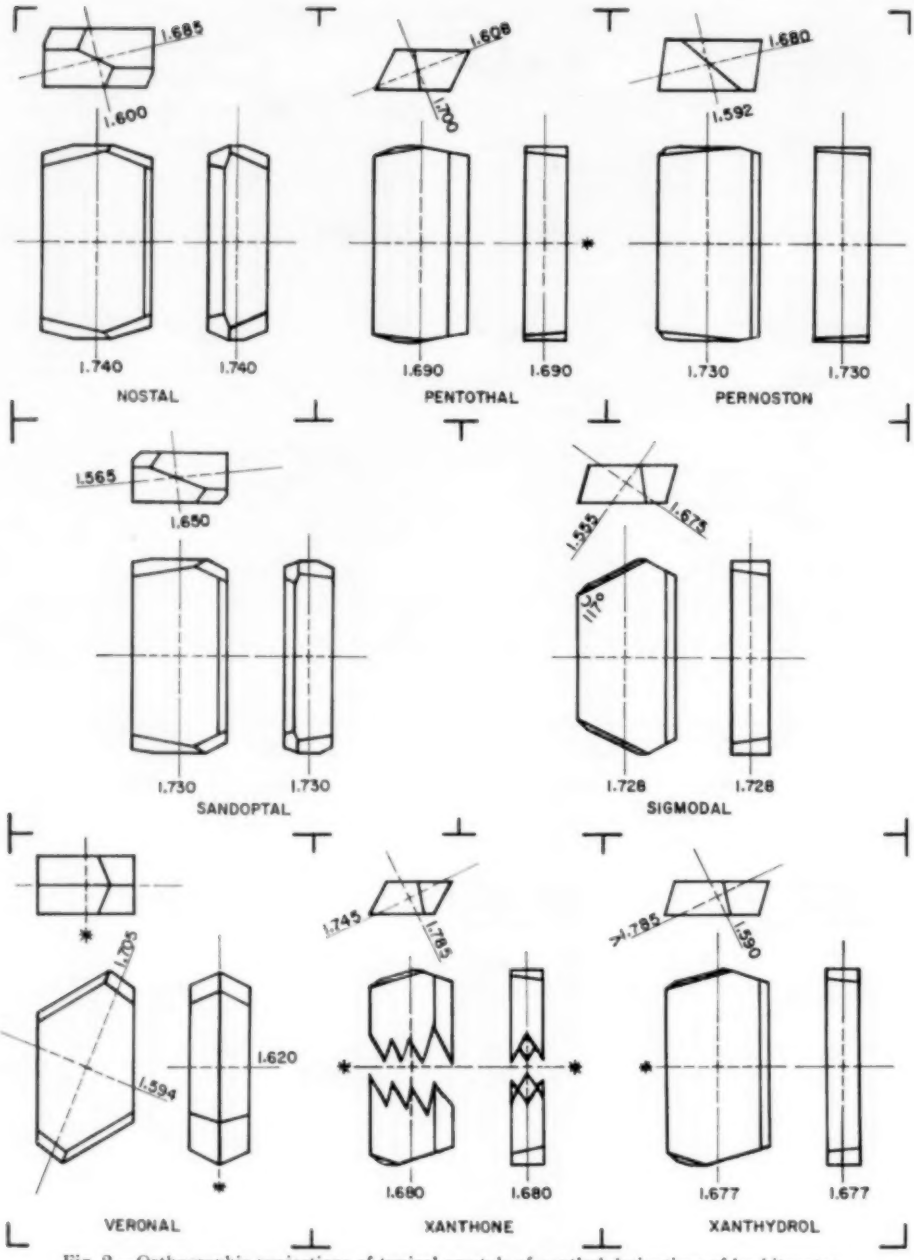

Fig. 2.—Orthographic projections of typical crystals of xanthyl derivatives of barbiturates.

ing fibrous crystals, twinned along the long axis, and showing very few top views. The variable index across the crystal in its usual orientation varied between 1.757 and 1.774. Xanthydrol has, in many respects, the same general appearance as xanthone; however, xanthydrol formed flatter and shorter crystals than xanthone and the optical properties distinguished the two compounds.

Xanthyl derivatives of Alurate, Dial, Nostal, Pernoston, Sandoptal and Veronal have similar prismatic habits, but are easily differentiated by other crystallographic properties.

TABLE III.—APPARENT PROPERTIES OF XANTHYL DERIVATIVES OF SOME BARBITURATES FROM MOST FREQUENTLY OBSERVED ORIENTATION

Xanthyl Derivative	Crystal Habit	Optical Orientation	Extinction Angle, Degrees	Refractive Indexes	
Alurate	Prismatic	Inclined Obtuse	0	Variable	1.725
Amytal	Acicular	Acute	0	1.664	1.730
Butisol	Tabular	Inclined Acute	0	Variable	1.716
Cyclopal	Prismatic	Inclined Obtuse	0	Variable	1.740
Dial	Prismatic	Inclined Obtuse	0	Variable	1.740
Ipral	Tabular	Inclined Obtuse	43	Variable	Variable
Luminal	Acicular	Acute	32	1.696	1.706
Neonal	Tabular	Obtuse	33	1.560	1.645
Nostal	Prismatic	Inclined Acute	0	Variable	1.740
Pentothal	Tabular	Inclined Obtuse	0	Variable	1.690
Pernoston	Prismatic	Inclined Acute	0	Variable	1.730
Sandoptal	Prismatic	Inclined Optic Normal	0	Variable	1.730
Sigmodal	Tabular	Inclined Acute	0	Variable	1.728
Veronal	Prismatic	Optic Normal	21	1.594	1.705
Xanthone	Acicular	Inclined Obtuse	0	1.680	Variable
Xanthydrol	Tabular	Inclined Optic Axis	0	1.677	Variable

The xanthyl derivative of Luminal presented a columnar or acicular appearance with twinned cleavage lines parallel to the *c* crystallographic axis. Consequently, most of these twinned crystals did not become extinct under crossed nicols. Single crystals were difficult to find, and these showed crossed dispersion.

The xanthyl derivative of Pentothal crystallized out in yellow, lath-shaped tablets which showed no pleochroism.

Some refractive indexes are reported as variable which is due to the reason explained above. The following are approximate values of some of the intermediate indexes taken from the crystals in their most frequently occurring orientations: xanthydrol, >1.785; xanthone, 1.757; xanthyl derivatives of, Alurate, 1.640; Butisol, 1.625; Cyclopal, 1.645; Dial, 1.630; Ipral, 1.617 and 1.679; Nostal, 1.680; Pentothal, 1.620; Pernoston, 1.675; Sandoptal, 1.570; and Sigmodal, 1.600.

Derivatives which did not form crystals suitable for study were: Delvinal, Nembutal, Ortal, Phanodorn, and Seconal.

The following barbiturates did not form xanthyl derivatives: Evipal (hexobarbital) and Mebaral (mephobarbital), Winthrop Laboratories, Inc.; Mosidal (methallatal) and Gemonil (metharbital), Abbott Laboratories; and Surital (thiamylal), Parke, Davis and Company.

SUMMARY AND CONCLUSIONS

A procedure for the preparation of xanthyl derivatives of barbiturates suitable for crystallographic study has been presented. The optical crystallographic properties of fourteen xanthyl derivatives of barbiturates and the micromelting points of these same derivatives and five additional ones are presented. The optical crystallographic properties of the xanthyl derivatives serve well as a means of identifying the fourteen barbiturates.

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The Biological Properties of Chlorindanol, A New Antiseptic Agent*

By JOHN HAYS BAILEY, FREDERICK COULSTON, and D. A. BERBERIAN

7-Chloro-4-indanol in concentrations of 1:1,000–1:3,000 is rapidly lethal to vegetative bacteria, *Trichophyton sp., C. albicans, E. histolytica* cysts and trophozoites, *T. vaginalis*, and spermatozoa *in vitro*. Systemic toxicity is low; it is well tolerated by skin, eyes, genital mucosa; it is nonallergenic; it appears to be suitable for use as topical antiseptic or sanitizer.

CHLORINDANOL, i. e., 7-chloro-4-indanol, was synthesized by Royal A. Cutler (1) of the Chemistry Division of the Sterling-Winthrop Research Institute. The structural formula is:

Chlorindanol is a crystalline substance with a molecular weight of 168.6 and m. p. 87.0–89.0° (corr.). It has limited solubility in water but is soluble in dilute alkali and in 95% alcohol to 5% w/v. The antimicrobial activity, toxicity, and irritation thresholds have been studied and are summarized below.

EXPERIMENTAL

The antimicrobial activity of chlorindanol was determined by inoculating representative species of bacteria and fungi into liquid culture media containing varied concentrations of the compound. The lowest concentration of compound which suppressed visible growth, when incubated (for eighteeen hours in the case of most bacteria or for ten days in the case of fungi, yeast-like organisms, and tubercle bacilli), was designated as the 'static concentration. Cultures which showed 'stasis were subcultured into twice the original volume of fresh medium and incubated for an additional forty-eight hours or fourteen days. The minimum drug concentration which prevented growth when subcultured was designated the 'cidal concentration.

Antibacterial Tests.—To a series of tubes containing respectively 3.0, 3.25, 3.5, and 3.75 ml. of culture medium was added, respectively, 1.0, 0.75, 0.5, and 0.25 ml. of a 5 mg./ml. solution of chlorindanol. Dilutions of 1:10 and 1:100 of the drug solution were added in a like manner to replicate series of tubes containing media. Each tube was then inoculated with 1.0 ml. of 1:1,000 dilution in broth of an eighteen to twenty-two-hour broth culture of the test organism, resulting in a series of tubes containing concentrations of chlorindanol ranging from 1.0 to 0.0025 mg./ml. contained in a final volume of 5 ml. per tube. End points were determined more exactly by repeating the test with drug concentrations varied at closer intervals within an appropriate range. The procedure was modified in the case of *Clostridium perfringens* by doubling all volumes to result in a final volume of 10 ml. per tube and the culture medium used was Bacto A-C medium. In the case of *Mycobacterium tuberculosis* the medium used was Youmans' liquid medium and the inoculum consisted of a suspension of tubercle bacilli harvested from Petragnani's medium and adjusted to provide 0.1 mg. per ml. For all other bacteria, tryptose phosphate broth (Bacto) was used, with addition of 2% normal horse serum for growth of streptococci or pneumococci.

Antifungal Tests.—These were performed in the manner described above except that the inoculum consisted of spores prepared by the method of the A. O. A. C. (2) and maltose peptone broth was used as the medium; incubation was at 25° for ten days. In the case of *Candida albicans* the inoculum consisted of a 1:1,000 dilution of a seventy-two hour culture in maltose peptone broth.

Germicidal Efficiency of Chlorindanol.—This was determined as the minimum period of exposure at room temperature required to kill the test organism exposed to given concentrations of germicide. In this procedure, 0.5 ml. of an eighteen to twenty-two-hour broth culture was added to 5 ml. of an aqueous solution of chlorindanol and rapidly mixed. At the end of various periods of exposure, 0.05 ml. of the test mixture was removed to 10 ml. of F. D. A. broth containing 0.7 mg. of lecithin per ml. of medium as an inactivator, and incubated for forty-eight hours at 37°. Absence of visible growth was taken to mean effective germicidal action within the exposure time represented by the sample.

The results of tests for antibacterial activity are shown in Table I. Chlorindanol was bacteriostatic and bactericidal for the 20 species of Gram-negative bacteria and the 10 species of Gram-positive bacteria. In general, chlorindanol was somewhat more active against Gram-negative than against Gram-positive bacteria. It is noteworthy that the difference between bacteriostatic concentration and bactericidal concentration was always small and in a number of instances the values were identical, suggesting that the action of the compound was rapid even at limiting dilutions. Chlorindanol was ineffective against spores of *Bacillus subtilis*, but it was effective against a strain of *C. perfringens* which is meagerly sporogenic. In terms of dilution, vegetative forms of all the species and strains tested were susceptible to chlorindanol 1:2,500 (one strain of *Pseudomonas*) to 1:25,000 (*M. tuberculosis*).

The germicidal efficiency of chlorindanol is shown by the data presented in Table II. *Staphylococcus aureus* 209 was killed within seven and one-half

* Received August 13, 1958, from the Biology Division, Sterling-Winthrop Research Institute, Rensselaer, N. Y.

TABLE I.—ANTIBACTERIAL ACTION OF CHLORINDANOL FOR GRAM-NEGATIVE AND GRAM-POSITIVE ORGANISMS

	Minimum Concentration Required, mg./ml.	
	'Stasis	Kill
Gram-negative		
Aerobacter aerogenes	0.2	0.2
Brucella abortus 11192	0.03	0.07
B. melitensis L-1	0.05	0.1
B. suis 1744	0.06	0.1
Desulfovibrio desulfuricans 7757	0.03	0.07
Escherichia coli 198	0.07	0.09
E. coli 4157 G	0.06	0.08
Eberthella typhi Hopkins	0.05	0.07
Klebsella pneumoniae 48	0.1	0.1
Pasteurella boviseptica Harvard	0.05	0.07
Proteus vulgaris 9920	0.1	0.1
P. mirabilis 9921	0.1	0.1
Pseudomonas aeruginosa N.O.	0.2	0.4
P. aeruginosa 211	0.4	0.4
Salmonella enteritidis 588	0.06	0.08
S. schottmuelleri	0.07	0.1
S. paratyphi 9150	0.07	0.1
Shigella dysenteriae Shiga.	0.05	0.07
S. paradysenteriae	0.06	0.07
Vibrio cholerae 23	0.03	0.04
Gram-positive		
Bacillus subtilis	0.05	>1.0
Clostridium perfringens Mich.	0.06	0.1
Diplococcus pneumoniae I	0.03	0.05
D. pneumoniae II	0.03	0.05
Mycobacterium smegmatis	0.04	0.05
M. tuberculosis H37RV	0.02	0.04
Staphylococcus aureus 209	0.06	0.15
S. aureus SWR1	0.06	0.06
Streptococcus faecalis 10C1	0.06	0.07
S. pyogenes C203	0.09	0.2
S. viridans 9811	0.08	0.15

minutes by a dilution of 1:1,500 at room temperature. *Eberthella typhi* Hopkins was the most susceptible of the organisms tested by this procedure. Relatively resistant strains of *P. aeruginosa* were killed by exposure to 1:1,000 for five minutes.

Table III shows that chlorindanol is effective *in vitro* in low concentrations against *Candida albicans* and a variety of fungi when exposure is prolonged. In a fungicidal efficiency test, it was demonstrated that *C. albicans*, *T. mentagrophytes*, and *A. niger* were killed by exposure to 1:1,000 and 1:2,000 dilutions for five minutes and sixty minutes, respectively (Table IV).

Conventional phenol coefficient values were determined for chlorindanol by the so-called F. D. A. procedure, using several species of bacteria as the test organisms. These values are summarized in Table V. These values were not significantly reduced when the subcultures were made into lecithin inactivator broth.

Chlorindanol 1:5,000 was amebacidal for *Endamoeba histolytica* when incorporated in Hansen's egg infusion medium, inoculated with the sediment from a pool of rich cultures, and incubated at 36° for thirty hours; a 1:10,000 dilution reduced the number of amebae per drop of sediment from 590 to 9, and 1:20,000 dilution was ineffective. A 1:2,500 dilution killed a heavy inoculum of cysts of *E. histolytica* in thirty minutes but not in ten minutes exposure.

Chlorindanol was tested for trichomonacidal activity by mixing various dilutions in isotonic saline solution with cecal content from hamsters containing approximately 3 million trichomonads (*Trichomonas muris* and *T. minuta*) per ml. Chlorindanol 1:2,000 immobilized all trichomonads in an average time of one minute and six seconds, 1:3,000 killed in three minutes, 1:4,000 killed in twelve minutes and forty-two seconds, and 1:5,000 killed in eighteen minutes and thirty seconds. When chlorindanol was added to rich cultures of *Trichomonas vaginalis* of human origin, in Johnson's medium (3), a dilution of 1:2,000 completely immobilized all trichomonads in less than one minute, 1:4,000 immobilized in two minutes, and 1:8,000 immobilized in thirteen minutes when immobilization time was measured from the moment of mixture of the culture with an equal volume of chlorindanol.

The action of chlorindanol on human spermatozoa was determined on two specimens of semen, from different patients, received in a viable condition from a collaborating physician. In accordance with the procedure of Brown and Gamble (4), a small quantity of semen was drawn into a capillary pipet, followed by an equal volume of drug dilution. The material was thoroughly mixed on a microscope slide and sealed under a cover glass. Final dilutions of 1:2,000, 1:3,000, 1:4,000, 1:5,000, 1:6,000, 1:8,000, and 1:10,000 chlorindanol were tested. The rate of immobilization was determined by continuous observation under the microscope. There was some variation between replicate samples, obviously due to variations in uniformity of mixing. However, it is clear that chlorindanol is a highly effective spermicide. The dilutions of 1:2,000 and 1:3,000 immobilized immediately; 1:4,000 immobilized in an average time of two minutes and thirty seconds, and three minutes; 1:5,000 immobilized in ten minutes and forty-eight seconds and eleven minutes and twelve seconds (range 3-18 and 3-25 minutes); 1:6,000 immobilized in twenty-three minutes and 1:8,000 in fifty-three minutes; 1:10,000 was not completely effective in three hours.

SAFETY EVALUATION OF CHLORINDANOL

The systemic toxicity of chlorindanol and its effect on tissues have been studied under a variety of experimental conditions as summarized below.

Acute toxicity (5).—The toxicity of single graduated doses of chlorindanol was determined by administration either orally, intravenously, or intraperitoneally to groups of ten male Swiss (Webster) albino mice weighing 22 ± 2.0 Gm. For oral and intraperitoneal medication the drug was prepared as a 4% stock solution in dilute NaOH and adjusted to pH 10.0. Appropriate aqueous dilutions were prepared to provide the desired dose levels in a volume of 0.01 ml./Gm. of body weight; oral administration was by stomach tube. For intravenous administration a 1.0% solution of chlorindanol was prepared and injection into the tail vein was made at a constant rate of 1.0 ml. per minute. The animals were observed for symptoms of acute toxicity over a period of several hours following medication and for delayed symptoms over the seven days following medication.

TABLE II.—GERMICIDAL EFFICIENCY OF CHLORINDANOL AT ROOM TEMPERATURE

Test Organism		Maximum Dilution Killing, Min. ½	1	2½	5	7½	10	15
S. aureus 209	a)	1:1,000	1:2,000	1:2,500	...	...	...	...
	b)	...	...	...	1:1,000	1:1,500	1:1,500	1:1,500
E. typhi Hopkins		1:1,000	1:3,000	1:3,000	1:4,000	1:4,000	1:4,000	1:4,000
Es. coli (2 strains)		...	...	...	1:3,000	...	1:3,500	1:4,000
P. aeruginosa 211		...	...	...	1:1,000	...	1:1,500	1:1,500
P. aeruginosa N.O.		...	...	...	1:1,000	...	1:1,500	1:1,500
M. tuberculosis H 37 Rv[a]		...	...	...	1:1,000	...	1:2,500	...

[a] Lethal effect confirmed by guinea pig inoculation.

TABLE III.—ANTIMICROBIAL ACTION OF CHLORINDANOL FOR CERTAIN YEAST-LIKE ORGANISMS AND FUNGI

	Minimum Concentration Needed, mg. ml. 'Stasis	Kill
Aspergillus niger	0.025	0.3
Candida albicans	0.03	0.03
Pityrosporum ovale	0.05	0.05
Trichophyton gypseum	0.025	0.03
T. interdigitale	0.025	0.025
T. mentagrophytes	0.0125	0.025

TABLE IV.—FUNGICIDAL EFFICIENCY

Organism	Min. Required to Kill—1:1,000	1:2,000	1:4,000
C. albicans	<5	<5	30
T. mentagrophytes	<5	<5	<5
A. niger	<5	<5	60

TABLE V.—PHENOL COEFFICIENT VALUES OF CHLORINDANOL

	Average P. C. at 20°—Without Serum Av. P. C.	Av. Limiting Diln.	With 10% Serum P. C.	Av. Limiting Diln.
S. aureus 209	27.0	1625	12.2	733
E. typhi Hopkins	46.4	4187	27.7	2,500
Es. coli 198	43.7	3500	25.8	2,333
Es. coli 4157 G	53.0	4166	22.2	2,000
Sal. enteritidis 588	56.2	4500	22.2	2,000
P. aeruginosa 211	21.8	1750	12.9	1,000
P. aeruginosa N.O.	19.3	1550	14.2	1,000

As a measure of toxicity, the LD_{50} was calculated by the method of Miller and Tainter (6) at twenty-four hours and at seven days after medication.

The LD_{50} values obtained are presented in Table VI. The symptoms of acute oral toxicity consisted of slight to severe respiratory depression and general depression, related to the magnitude of the dose. The deaths occurred within two to fifteen hours after medication. Intravenous injection of toxic doses caused hyperexcitability, tonic and clonic convulsions, and death due to respiratory arrest within one to ten minutes after injection; there were no delayed deaths. Intraperitoneal injection of toxic doses caused severe general depression and the deaths occurred sporadically over a period of several days. The values given in Table VI indicate that chlorindanol is not a highly toxic compound.

TABLE VI.—ACUTE TOXICITY OF CHLORINDANOL FOR 22-GM. MICE

Route of Administration	LD_{50} ± S. E., mg./Kg.—at 24 hrs.	at 7 days
Intravenous	48 ± 2	48 ± 2
Intraperitoneal	188 ± 30	96 ± 22
Oral[a]	960 ± 52	920 ± 35

[a] Administered as an alkaline solution at pH 10.0. When chlorindanol was administered as a suspension in 1% gum tragacanth for five consecutive days the LD_{50} for mice was greater than 1,585 mg./Kg./day and for rats was calculated to be 1,650 ± 265 mg./Kg./day.

Subacute toxicity.—Two types of subacute toxicity tests were carried out. The determination of an LD_{50} on the basis of daily doses administered by stomach tube for five consecutive days, followed by observation for seven subsequent days, is particularly useful for bringing out any tendency for cumulative delayed toxic manifestations. Secondly, a three weeks toxicity test carried out at relatively high dose levels is useful in providing a measure of the degree of tolerance to be expected from chronic intake or absorption of the drug.

Five-day subacute toxicity tests were carried out both in mice and in rats (Sprague-Dawley strain). Chlorindanol was administered to groups of five male albino mice as single daily doses of 795 and 1,585 mg./Kg. for five consecutive days. The drug was administered as a suspension in 1.0% gum tragacanth solution. A control group received only the vehicle for five days. Hematological studies were carried out on all surviving mice one day before they were sacrificed and autopsied. The mice which received 795 mg./Kg. daily developed moderate respiratory and general depression within twenty to thirty minutes after each dose. The depression persisted for four to five hours after the first dose and was of progressively shorter duration thereafter. There were no deaths at this dose level and body weight gain did not differ significantly from that of the controls. All of the mice which received the daily dose of 1,585 mg./Kg. showed severe respiratory and general depression within twenty to thirty minutes after medication; these animals remained prostrate for four to five hours after the first dose but the symptoms became progressively less severe. At the high dose level one mouse died about 12 hours after the first dose but all others survived with only moderate weight loss during the medication period and weight was gained at a normal rate after the last dose. The blood studies did not show any ab-

normal deviation in number or morphology of leucocytes or erythrocytes, and pathological changes attributable to the medication were not found at autopsy.

Chlorindanol was administered orally once daily for five consecutive days to three groups of ten male albino rats at dose levels of 800, 1,600 and 3,200 mg./Kg. The drug was suspended in 1.0% gum tragacanth. A control group of ten rats received only the vehicle. The animals were observed for seven days after the last dose and blood studies were carried out on the control rats and the seven surviving rats of the 1,600 mg./Kg. group on the sixth post-medication day; all survivors were sacrificed and autopsied on the seventh day.

All rats which received 800 mg./Kg. daily for five days were normal in appearance and behavior throughout the experiment. Four of the ten rats in the 1,600 mg./Kg. group developed blood tinged nasal exudate and three of them died (two on the third day and one on the fourth day of medication). All of the rats on the high dose level (3,200 mg./Kg.) developed bloody secretions, diarrhea, and died by the end of the fourth day of medication. The body weight gain of the rats on the low dose was slightly depressed, and the 1,600 mg./Kg. dose caused moderate depression of weight gain during the medication period; all survivors gained at a normal rate as soon as medication was stopped. There was no significant deviation from normal leucocyte or erythrocyte counts, differential count or hemoglobin concentration of the control rats or seven survivors of the 1,600 mg./Kg. group. Significant gross or histopathological changes were not found on autopsy of all surviving rats. The 5-day LD_{50} of chlorindanol in rats was calculated to be 1,650 ± 265 mg./Kg. per day. It is apparent from this study that chlorindanol, orally, is less readily absorbed, and consequently less toxic, when administered as a suspension than when administered as an alkaline solution.

Three-Weeks Subacute Toxicity Test in Rats.—Chlorindanol was administered orally, by stomach tube, to three groups of ten male albino Sprague-Dawley rats in single daily doses of 200, 400, and 800 mg./Kg. A total of 18 doses was administered over a period of twenty-one days. The drug was given as a suspension in 1.0% gum tragacanth. A control group of ten rats received only the vehicle. The animals were weighed three times a week and carefully observed for signs or symptoms of toxicity. Hematological studies were carried out on all of the animals four days before the end of the experiment. At the end of the experiment all animals were sacrificed with ether, the thoracic and abdominal viscera were examined for gross lesions, and portions of each organ were fixed in Zenker-formalin for histological study.

All of the animals remained normal in appearance and behavior throughout the experiment. One rat on the low dose died of intercurrent pneumonia on the tenth day. The average growth rate of the rats on the low dose was similar to that of the control group throughout the experiment. The growth of the rats on the 400 and 800 mg./Kg. dosage was depressed in relation to size of the dose; body weight was 11.3% and 38% less than the control group at the end of the experiment. The blood studies did not show any abnormal deviation in the total erythrocyte or leucocyte count, in hemoglobin concentration, differential count, or hematocrit. Pathological changes in organs or tissues, attributable to medication with chlorindanol, were not found.

Irritation Studies.—Experiments were carried out to determine the capacity of chlorindanol to cause toxic irritation to skin, eyes, and genital mucosa of rabbits. The procedures used were those of Draize, Woodard, and Calvery (7) and the severity of ocular inflammation was scored by the method of Hoppe, *et al.* (8).

Skin irritation tests were carried out on 14 rabbits, of which 12 were treated with chlorindanol and two were treated with control solutions. The area of the clipped back and sides of each animal was marked off into quadrants. The skin of alternate quadrants was abraded by means of two pairs of intersecting scratches; each scratch was one centimeter long. Each test area was covered with a 4 x 4 cm. sterile gauze pad secured to the skin by means of adhesive tape. An equal number of abraded and intact areas of skin were treated with powdered crystalline chlorindanol and with a 2.0% solution of the drug in 46% polyethylene glycol (PEG-400). Controls received only the vehicle. The test materials were introduced as uniformly as possible under the pads. The treatment consisted of 0.5 Gm. of pure chlorindanol powder or 0.5 ml. of the 2.0% solution. After application of the medication, the entire trunk of each rabbit was wrapped in polyethylene sheet and a heavy cloth. The wrappings and the pads were removed after twenty-four hours for examination and scoring; additional observations were made at seventy-two hours and seven days. For evaluation, the scores at twenty-four and seventy-two hours were averaged separately and the derived values were then averaged to give the primary irritation index.

According to Draize, *et al.* (7), "compounds producing combined averages (Primary irritation indexes) of 2 or less are only mildly irritating whereas those with indexes from 2–5 are moderate irritants, and those above 6 are considered severe irritants." In the experiment described above, the combined average for chlorindanol powder was zero, for the 2.0% solution in PEG-400 the index value was 0.05, and for the vehicle control the index was zero. It is concluded that under the conditions of the experiment, chlorindanol is not a primary irritant for the skin of the rabbit.

Eye irritation tests were carried out according to Draize, *et al.* (7), and Hoppe, *et al.* (8). The concentrations of chlorindanol tested were 0.5, 0.75, 1.0, and 1.5% in 67% PEG-400. Twenty-two rabbits were used for the experiment, five for each dose level and two for vehicle controls. One-tenth milliliter of the test solution was instilled into the conjunctival sac of the right eye of each rabbit. The condition of the medicated eye was compared with that of the unmedicated left eye at twenty-four, forty-eight, seventy-two, and ninety-six hours, and at seven days. The results indicated that the 0.5% solution was completely tolerated; three of five animals receiving 0.75% solution showed transitory mild hyperemia which disappeared within twenty-four hours; the two higher doses caused moderate hyperemia which was apparent at twenty-four, forty-eight, and ninety-six hours but not at one hundred-twenty hours; partial cloading of the cornea was observed in two rabbits of the 1.0% group and four of five rabbits of the 1.5% group but all eyes were normal by

the seventh day. The control solution of the 67% PEG-400 did not cause any inflammation. Under the conditions described, chlorindanol 0.5% was nonirritating, 0.75% was very mildly irritating, 1.0%, and 1.5% were only moderately irritating. Chemosis and discharge were minimal at the higher concentration. In an additional experiment on five rabbits, a 2.0% solution of chlorindanol in 46% PEG-400 was rated as only moderately irritating and all eyes were clear at seven days; the maximum average total score, at forty-eight and seventy-two hours, was 10.2 of a maximum possible score of 20.

An experiment was carried out to determine the effect of chlorindanol on the penile mucosa of the rabbit. Three adult male albino rabbits were used to test the irritancy of 2.0% chlorindanol solution in 46% aqueous PEG-400. A fourth rabbit was used to test the vehicle, as a control. The penis and urethral orifice of each rabbit was thoroughly wetted with 0.2 cc. of test solution, which was not removed. Observations were made at one hour, twenty-four, and forty-eight hours after medication. There was no visible evidence of inflammation after application of 2.0% chlorindanol in PEG.

Allergenicity.—An experiment was carried out in guinea pigs to test the allergenicity of chlorindanol in accordance with the procedure of Draize, *et al.* (7). Seven male guinea pigs, ranging from 325 to 450 Gm. in weight, were used. Five guinea pigs were prepared by a series of ten intradermal injections of an 0.1% solution of chlorindanol in 10% PEG-400, and two guinea pigs received similar "sensitizing" injections of 10% PEG-400. The injections were made intradermally, three times a week until a total of ten had been completed. The first injection was a single dose of 0.05 ml. followed by nine injections of 0.1 ml. At the time of each injection, the area of any resulting erythema and induration was recorded. Two weeks after the last preparative dose, each guinea pig was challenged for evidence of induced hypersensitivity by an intradermal injection of 0.05 ml. of a fresh 0.1% solution of chlorindanol in PEG-400, and for control purposes each animal also received 0.05 ml. of 10% PEG-400. The sites of injection were observed for immediate reaction, and again twenty-four hours after injection. Areas of erythema were measured for comparison with the response to the first preparative injection. It was not possible to distinguish between the wheals produced by the PEG-400 alone and PEG-400 containing chlorindanol, or between the primary response and the challenge response. It was concluded that chlorindanol is not an allergenic substance under the conditions described.

Solutions of 0.5%, 1.0%, and 2.0% chlorindanol in 46% aqueous PEG-400 did not modify the course of healing of incisions 1 cm. in length made into the dermal layer of skin of six adult albino rabbits. Each incision was covered with a 4-cm. square sterile gauze pad and 0.5 ml. of test solution was applied daily for three days. For the first three days the solution was introduced under the pad and the trunk of the animal was securely wrapped. After the third day the wrappings were omitted and pads containing test solution were held in contact with the wound for one minute twice daily for seven days, at which time the experiment was terminated. During the course of observation there was no evidence of secondary infection or of irritation attributable to the solutions applied.

As the result of the experiments described above, it is concluded that the intrinsic toxicity of chlorindanol is low, it is nonallergenic, and it is well tolerated by skin, eyes, or genital mucosa.

SUMMARY

Chlorindanol 1:1,000 to 1:3,000 is rapidly lethal to vegetative bacteria, *Trichophyton* sp., *Candida albicans*, *Endamoeba histolytica* cysts and trophozoites, *Trichomonas vaginalis*, and spermatozoa *in vitro*. Oral toxicity is low, but is somewhat greater when administered in alkaline solution (LD_{50} for the mouse = 920 ± 35 mg./Kg.) than when suspended in gum tragacanth (LD_{50} for five consecutive daily doses = >1,500 mg./Kg./day). In a three weeks toxicity test, rats completely tolerated daily oral doses of 200 mg./Kg. Dose levels of 400 and 800 mg./Kg. caused some retardation of weight gain but there were no deaths. Extensive irritation studies showed that chlorindanol solutions of less than two per cent in PEG-400 were well tolerated by skin, conjunctiva, and genital mucosa of rabbits, and were also shown to be nonallergenic for guinea pigs. A two per cent solution in forty-six per cent PEG-400 did not interfere with healing of wounds. The properties of chlorindanol indicate that it is suitable for topical antiseptic or sanitizing purposes.

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The Analysis of Digoxin Preparations*

By ALBERT E. H. HOUK, THOMAS G. ALEXANDER, and DANIEL BANES

Methods are proposed for the analysis of crystalline powders, tablets, injections, and elixirs containing digoxin. The glycoside is assayed by means of an improved *m*-dinitrobenzene reagent, and its purity is determined by chromatographic and colorimetric tests.

AN EARLIER REPORT from this laboratory (1) described a procedure employing *m*-dinitrobenzene in weakly alkaline solution for the colorimetric determination of digoxin. Further studies on the chromogenic reaction showed that small quantities of water in the alcoholic reagent profoundly influenced both the purity and the intensity of the color developed. That reagent produced colors with absorption maxima at 480 and 600–615 mμ. Use of the reagent proposed below yielded more intense colors with a single peak in the visible region at 620 mμ. The colors attained their maximum absorbances in about ten minutes at 25° under the conditions of the test, with only slight deviation from the Beer-Lambert law.

PROPOSED METHODS OF ANALYSIS

Alkaline Dinitrobenzene Reagent

(*a*) Prepare a 5% solution of *m*-dinitrobenzene in benzene, and store in a glass-stoppered brown glass bottle. (*b*) Mix 1 ml. of 10% tetramethylammonium hydroxide solution with 140 ml. of absolute alcohol, titrate a portion with 0.01 *N* hydrochloric acid, and adjust the remainder to 0.008 *N* with absolute alcohol. Just prior to use, mix 60 ml. of (*a*) with 40 ml. of (*b*).

Assay Procedure

Sample Preparation.—(*a*) *Crystalline Digoxin.*—Prepare an alcoholic solution containing 125 μg. of digoxin per ml. Transfer 10.0 ml. to a separator, add 50 ml. of water and 1 ml. of 2 *N* sulfuric acid, and extract with three 30-ml. portions of chloroform. Wash each chloroform extract in a second separator by shaking with 10 ml. of water and 1 Gm. of ground anion-cation exchange resin,[1] and filter through a pledget of cotton moistened with chloroform into a 100-ml. volumetric flask. Dilute to the mark with chloroform, and mix well. This solution is the *Assay Preparation.*

(*b*) *Elixirs and Injections.*—Transfer an aliquot containing 1.25 mg. of digoxin to a separator, and proceed as directed under *Crystalline Digoxin* beginning with the words: "Add 50 ml. of water and 1 ml. of 2 *N* sulfuric acid . . ."

(*c*) *Tablets.*—Weigh into a 100-ml. beaker an accurately measured portion of powdered tablets equivalent to 1.25 mg. digoxin. Add 10 ml. of boiling alcohol, and stir. Cover with a watch glass, and allow to stand for twenty minutes at 60° with frequent stirring. Cool, wash into a separator quantitatively with 30 ml. of chloroform and 50 ml. of water, add 1 ml. of 2 *N* sulfuric acid, and proceed as directed under *Crystalline Digoxin* beginning with the words: "extract with three 30-ml. portions of chloroform. . . ."

Standard Preparation.—An alcoholic solution containing 25.0 μg. of U. S. P. Reference Standard Digoxin per ml.

Colorimetric Determination.—Pipet 5.0 ml. of the *Standard Preparation* and 10.0 ml. of the *Assay Preparation* into similar Erlenmeyer flasks, and evaporate to dryness on the steam bath with the aid of a current of air. Cool and add to each dry residue 5.0 ml. of freshly prepared alkaline dinitrobenzene reagent. Let stand five minutes at a temperature not exceeding 30°, with frequent mixing. Determine the absorbances of the developing blue colors relative to the reagent blank at 620 mμ at one-minute intervals, using matched 1-cm. cells and a suitable spectrophotometer. Record the maximum absorbance of the aliquot from the *Assay Preparation* as *A* and that from the *Standard Preparation* as *S*. The quantity, in mg., of $C_{41}H_{64}O_{14}$ in the *Assay Preparation* is given by the expression $1.25\ A/S$.

Other Digitoxosides.—Pipet 20.0 ml. of the *Assay Preparation* and 10.0 ml. of the *Standard Preparation* into separate 50-ml. Erlenmeyer flasks and evaporate to dryness on the steam bath with the aid of a current of air. Cool, add 4.0 ml. of Keller-Kiliani reagent (1) to each beaker and mix thoroughly. After ten minutes, determine the absorbances of the sample and standard at 590 mμ relative to the reagent blank at five-minute intervals. Record the maximum absorbance of the sample as *A*, and that of the standard as *S*. The quantity in mg. of total digitoxosides calculated as digoxin in the *Sample Preparation* is $1.25\ A/S$. The difference between this value and that obtained in the *Assay Procedure* indicates the quantity of other digitoxosides in the *Sample Preparation*.

Chromatographic Identification

Materials.—(*a*) *Immobile Solvent.*—Dilute 35 ml. of U. S. P. grade formamide to 100 ml. with acetone and mix. Prepare fresh weekly.

(*b*) *Trichloroacetic Acid Spray Reagent.*—Dissolve 6 Gm. of trichloroacetic acid in 25 ml. of U. S. P. chloroform and mix. Prepare fresh daily. Prior to use as a spray, add 0.2 ml. of 30% hydrogen peroxide per 10 ml. of reagent (2).

(*c*) *Mobile Solvent.*—Just prior to use, shake 100 ml. chloroform with 3 ml. redistilled formamide. Filter the chloroform layer through a cotton pledget.

(*d*) *Standard Preparation.*—Prepare a solution containing 2.5 mg. U. S. P. Reference Standard Digoxin per ml. of alcohol-chloroform (1 + 2).

* Received August 21, 1959, from the Department of Health, Education, and Welfare, Food and Drug Administration, Bureau of Biological and Physical Sciences, Division of Pharmaceutical Chemistry, Washington, D. C.

[1] Rohm and Haas Amberlite MB-1, analytical grade, indicator-free, was found satisfactory.

(*e*) *Sample Preparation.*—Transfer 50 ml. of the *Assay Preparation* to a 125-ml. Erlenmeyer flask and evaporate to approximately 1 ml. on the steam bath with the aid of a current of air. Transfer the solution to a small weighing bottle with the aid of chloroform, and evaporate to dryness. Dissolve the residue in 0.25 ml. of alcohol-chloroform (1 + 2).

Procedure.—Arrange a suitable chamber for ascending chromatography (3) using Whatman No. 1, 8 x 8 inch filter paper impregnated with the *Immobile Solvent*. Spot separately 0.01 ml. of the *Sample Preparation* and *Standard Preparation*, in duplicate. Place the paper in the chamber, and permit the chromatogram to develop until the *Mobile Solvent* has moved to within an inch of the top. Remove the paper from the chamber and heat at 90° in a current of air for fifteen minutes. Remove the paper, spray with *Trichloroacetic Acid Spray Reagent*, and again heat at 90° for ten minutes. Examine the chromatogram under an ultraviolet lamp. The sample shows a single fluorescent spot, corresponding to that of the standard. (In order of decreasing R_f values, digitoxin gives a yellow-orange fluorescent spot; gitoxin, a yellow to blue spot; digoxin a blue spot; diginatin (4) and lanatoside C, blue spots near the starting line.)

DISCUSSION

The proposed method of assay was applied to U. S. P. Reference Standard Digoxin; U. S. P. Reference Standard Digitoxin; commercial samples of gitoxin, desacetyllanatoside C, and lanatoside C; two simulated tablet mixtures containing 0.0985% and 0.125% digoxin, respectively; two simulated injection mixtures, one containing 50% glycerol and the other 50% propylene glycol, and twenty commercial digoxin preparations of various types. The more polar members of the lanatoside C series yielded colors similar to that of digoxin with the reagent but desacetyllanatoside C was completely removed, and lanatoside C partially removed in the extraction process. Digitoxin and gitoxin yielded red colors in the procedure. The absorbance of the color due to digitoxin was about 1/5, and that of gitoxin about 1/20 of the absorbance due to digoxin at 620 mμ. Excessive quantities of these substances would be detected in the Keller-Kiliani assay and chromatographic test.

Analytical data obtained for U. S. P. Reference Standard Digoxin after extraction were identical with the values obtained for the unextracted standard in both colorimetric procedures. Recoveries of digoxin from simulated injections and tablet mixtures ranged from 97.4 to 100.5% of the quantities incorporated, and agreement between analysts and between replicate analyses demonstrated excellent reproducibility. It was necessary to use glassware scrupulously free of acid and base in performing the dinitrobenzene colorimetric test, since the alkalinity of the reaction mixture is critical. The ion exchange resin was introduced to eliminate interferences caused by stearate lubricants.

Assay values for digoxin and total digitoxosides in twenty commercial preparations are listed in Table I. The greatest difference between the two values (sample 16) was 7%, but these analyses were performed on different subsamples of tablets. In some cases the Keller-Kiliani value was as much as 4% lower than the *m*-dinitrobenzene assay, but this discrepancy may merely reflect the limits of error of the methods. The paper chromatograms showed no excessive quantities of contaminating steroids in any of the digoxin preparations.

TABLE I.—ASSAYS OF COMMERCIAL DIGOXIN PREPARATIONS

		Per Cent of Declared		
		Digoxin by *m*-Dinitrobenzene		Digitoxosides by Keller-Kiliani Test
Sample	Description	Analyst 1	Analyst 2	
1	Powder	100.1	99.0	100.3
2	Powder	95.4	94.0	100.5
3	Powder	95.4	94.4	98.0
4	Powder	94.0	..	..
5	Injection, 0.25 mg./ml.	94.0	94.1	..
6	Injection, 0.25 mg./ml.	91.8	91.9	..
7	Injection, 0.25 mg./ml.	91.4	92.0	..
8	Elixir, 0.05 mg./ml.	95.5	95.6	..
9	Tablets, 0.25 mg./tablet	99.5	99.8	101.0
10	Tablets, 0.25 mg./tablet	96.3	98.2	93.6
11	Tablets, 0.25 mg./tablet	95.2	97.8	95.4
12	Tablets, 0.5 mg./tablet	97.1	96.8	96.7
13	Tablets, 0.25 mg./tablet	94.0	96.2	93.1
14	Tablets, 0.25 mg./tablet	96.2	95.8	95.4
15	Tablets, 0.25 mg./tablet	95.2	95.2	95.6
16	Tablets, 0.25 mg./tablet	97.9	94.3	101.4
17	Tablets, 0.25 mg./tablet	90.5	90.3	92.5
18	Tablets, 0.25 mg./tablet	88.7	88.3	88.7
19	Tablets, 0.25 mg./tablet	86.2	89.6	85.2
20	Tablets, 0.25 mg./tablet	89.7	89.5	89.3

SUMMARY

Methods are proposed for the chemical identification and analysis of digoxin in commercial preparations. These methods have been applied successfully to crystalline powders, tablets, injections, and elixirs containing digoxin.

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The Effect of Mouthwashes on the Oral Flora*

By MORRIS OSTROLENK† and WILLIAM WEISS

A study has been made of the efficacy of several commercial mouthwashes representing as many classes of chemical agents. The method employed tends to represent, as closely as possible, the conditions of use. The selective antibacterial activity of the washes was measured in terms of "before" and "after" total bacterial counts, and the incidence of streptococci, staphylococci, fusoform bacteria, and lactobacilli. There is included a study of the length of time that elapses after the use of the mouthwashes before the number of bacteria in the oral cavity reaches the number present before washing. Data obtained from these studies indicate that each of the compounds significantly reduces the total bacterial count in the mouth, and that some deal selectively with microorganisms. Each mouthwash, however, leaves residues of active bacteria among those removed from the oral cavity by a rinse technique. It appears also that the effect of the compounds is of short duration.

THIS COMMUNICATION continues the report of the investigation that has been in progress for the purpose of developing specific *in vivo* bacteriological methods for the evaluation of mouthwashes (1, 2).

The data presented here deal with: (*a*) The numbers of several types of microorganisms that can be washed out of the mouth by a rinse technique, (*b*) the selective antibacterial activity of several oral "antiseptic" mouthwashes, and (*c*) the length of time that elapses after the use of "antiseptic" mouthwashes before the number of bacteria in the oral cavity reaches the number present before washing.

METHODS

Two experiments were undertaken for the purposes of this study. In the first, five commercial mouthwashes, designated *B*, *C*, *E*, *G*, and *H*, and a sterile physiological salt solution, *X*, used as a control, were tested. Six subjects participated in this experiment.

A subject was given, at hourly intervals, four 30-second rinses, referred to as washes 1, 2, 3, and 4. Each rinse consisted of a 20-cc. solution of 0.85% salt. Midway between the second and third rinses, he was given an oral rinse consisting of one of the antiseptic mouthwashes or of the control, another salt solution.

Following each rinse, the wash solutions were expectorated into sterile petri dishes, appropriately diluted, and immediately planted into six semidiagnostic culture media as follows: (*a*) semisolid thioglycollate shake agar tubes (2), incubated at 34° for forty-eight hours; (*b*) Chapman's streptococcus agar (3), incubated at 34° for forty-eight hours; (*c*) Chapman's staphylococcus agar (4), incubated at 37° for seventy-two hours; (*d*) Littman's yeast medium (5), incubated at 30° for ninety-six hours; (*e*) Spaulding-Rettger fusobacterium medium (6), incubated in anaerobic jars at 37° for ninety-six hours; and (*f*) Tittsler's lactobacillus medium (7),[1] incubated at 37° for seven days.

These media were tested successfully in preliminary studies undertaken to verify that each was selective for the group of microorganisms that it was designed to detect. Seventeen volunteer subjects participated in the preliminary studies. The oral cavity of each subject harbored the test organisms, in widely varying numbers, but in only six was yeast recovered.

Six of these 17 subjects were chosen for the experiment. The six included three, one of whom was yeast positive, with bacterial counts on thioglycollate agar of less than 100 million; and three, two of whom were yeast positive, with bacterial counts on thioglycollate agar of 150 million or more.

Each of the six subjects received the mouthwashes *B*, *C*, *E*, *G*, *H*, and *X* once a week for six weeks, in the pattern of a Latin square arrangement (2). This procedure was repeated during an additional six weeks.

The methods employed in the second experiment were essentially those used in the first with the following differences: (*a*) The antiseptic mouthwash (or control salt solution *X*) was given between the first and second hourly rinses instead of between the second and third; (*b*) four subjects participated and each received, on an average, two of eight mouthwashes, including the control, each week for eight weeks. Eight determinations were made on each compound. The mouthwashes employed, in addition to *X*, were *B*, *C*, *D*, *E*, *F*, *G*, and *H*. The mouthwashes offered the test subjects were selected at random, with the restriction that no subject received the same mouthwash twice in one week; and (*c*) the oral rinse solutions (washes 1, 2, 3, and 4) and the control *X* were planted, in duplicate, in thioglycollate shake agar tubes for total bacterial counts.

RESULTS

Table I gives the results obtained from washes 1 and 2 in the first experiment. Subjects 4, 5, and 6, chosen because their total bacterial counts on thioglycollate agar were in excess of 150 million, re-

* Received September 9, 1958, from the Division of Antibiotics, Food and Drug Administration, Department of Health, Education, and Welfare, Washington, D. C.

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[1] Canned whole tomatoes, filtrate, 200 cc.; distilled water, 800 cc.; tryptone, 5 Gm.; tryptose, 5 Gm.; yeast extract, 5 Gm.; agar, 12.5 Gm.; sodium azide 1:10,000. Adjust to pH 7.0 with sodium hydroxide, readjust with lactic acid to a final pH of 5.0.

tained this characteristic for the duration of the twelve-week test period. Their average first-wash bacterial counts were 163,489, and 225 million, respectively. Subjects 1, 2, and 3, with initial total bacterial counts of less than 100 million, showed first-wash averages of 89, 56, and 76 million for the same period. In addition, the three subjects selected as yeast negative and the three chosen as yeast positive remained, respectively, negative and positive throughout the twelve weeks.

In general, except for lactobacilli, bacterial counts rose between the first and second washes. It has been pointed out that this phenomenon is to be expected (2). The subjects consumed a meal sometime prior to each test and then ate no solids during the four-hour testing period. Eating is a mechanical degerming process; bacterial populations tend to decline after meals and then to return gradually to premeal levels.

Table II lists the third and fourth wash counts, expressed as a per cent of the second wash count (made thirty minutes prior to the application of the test solution), for each of the test compounds. These numerical estimates measure the activity of each of the test products, thirty and ninety minutes after administration.

A statistical analysis of variance was made separately for each test compound, for each organism group, and for both the thirty and ninety-minute intervals in which the antibacterial activity of each compound was compared with the control *X*.

Each of the five test products reduced significantly ($p < 0.05$) the total bacterial counts at both time intervals, in comparison with the reduction brought about by the mechanical action of the control salt solution *X*. There were similar significant reductions for each product in the streptococci counts.

Only compounds *E* and *G* reduced significantly the staphylococcus counts at the thirty-minute period, as compared with the cleansing action of the control. Mouthwash *G* was the only compound with a demonstrable antistaphylococcic effect at the ninety-minute measurement.

Only mouthwash *B*, and only for the time period of thirty minutes, gave a significant reduction in the yeast counts. Compounds *H* and *C* showed significant antibacterial activity thirty minutes after use in the fusoform bacterial counts, with only *H* giving prolonged activity for the additional hour until the ninety-minute measurement. Only *G* and *C* significantly reduced the lactobacilli counts, and each only at the thirty-minute period.

There were, for each of the counts, differences, often statistically significant, among the activities of the various test compounds. A study of Table II will show the hierarchies of the compounds with respect to their activities in each measurement.

The second experiment was designed to determine the length of time before total bacterial counts returned to normal following the use of seven commercial mouthwashes and a physiological salt-solution control. Results are given in Fig. 1.

The experiment shows that the logarithms of the bacterial counts obtained one-half hour, one and one-half hours, and two and one-half hours after the application of the mouthwashes lie approximately on straight lines. Recovery rates, expressed as a per cent increase over the preceding hourly wash bacterial counts, were: *C*, 15%; *B*, 30%; *G*, 33%; *H*, 41%; *E*, 49%; *D*, 56%; *F*, 66%; and the control, *X*, 26%. That is, on the average, the bacterial counts increased each hour by these percentages.

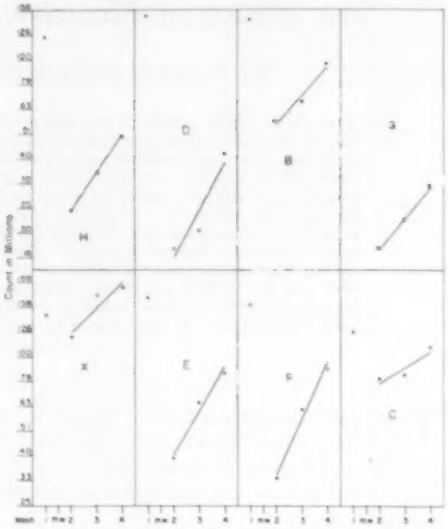

Fig. 1.—Duration of effect of test compounds.

On the basis of a statistical analysis, none of the recovery rates was significantly different from the others.

COMMENTS

In the evaluation of the *in vivo* antiseptic activity of mouthwashes, two measurements are of main significance. One of these is the count of microorganisms remaining in the oral cavity after the use of a compound, measured in terms of external recovery by some mechanical means. The technique employed here was an oral rinse.

It is evident that the five groups of microorganisms studied here occur at different numerical levels in the mouths of different individuals. Except for yeast, which has not been shown to vary appreciably with total bacterial counts, there is some variation in the population of group microorganisms related to the different total bacterial counts of individual subjects. On the basis of studies undertaken thus far (1, 2), in which approximately 200 subjects have participated, the total number of bacteria that can be removed from the mouth by rinsing ranges up to 500 million. No subject has yet been encountered, however, with a total count of less than 5 million following the use of an antiseptic mouthwash.

In the present study, the most effective product, *H*, permitted an average of 13.1% bacterial survival thirty minutes after its use. Within an hour and one-half after the use of this product, the average number of bacteria recoverable by a rinse was 28% of what it had been prior to washing. At this point, there was an average of 40 million recoverable bacteria present in the mouths of the test subjects.

Product *H* significantly reduced at the thirty and ninety-minute levels the number of streptococci and

TABLE I.—AVERAGE NUMBER OF BACTERIA RECOVERED FROM FIRST AND SECOND WASHES

Wash	Total Count[a] 1	2	Streptococci[a] 1	2	Staphylococci[b] 1	2	Yeast[b] 1	2	Fusobacteria[b] 1	2	Lactobacilli[b] 1	2
Subject 1	89	108	19	24	1.9	2.6	0	0	22.9	34.6	6.3	2.8
Subject 2	56	86	7	11	1.4	2.8	1.0	1.5	5.6	7.5	131.5	89.4
Subject 3	76	115	17	25	1.4	2.1	0	0	20.0	17.2	18.9	15.7
Subject 4	163	164	47	33	6.7	4.3	1.0	0.6	28.6	36.5	130.0	89.5
Subject 5	489	591	103	122	1.0	0.8	1.1	3.0	39.5	48.4	1840.0	2230.0
Subject 6	225	255	42	46	5.2	7.3	0	0	57.0	68.9	27.8	31.4
Average	137	173	28	33	2.2	2.7	1.0	1.4	23.4	28.6	68.5	54.0

[a] Counts in millions of bacteria. [b] Counts in thousands of bacteria.

TABLE II.—PER CENT RECOVERY OF MICROORGANISMS THIRTY AND NINETY MINUTES AFTER USE OF FIVE COMMERCIAL MOUTHWASHES AND A CONTROL

Compound	Percentage[a] of Second Wash Counts — Total Count 30 min.	90 min.	Streptococci 30 min.	90 min.	Staphylococci 30 min.	90 min.	Yeast 30 min.	90 min.	Fusobacteria 30 min.	90 min.	Lactobacilli 30 min.	90 min.
B	24	52	32	52	58	62	21	47	36	92	58	51
C	34	44	18	34	40	65	45	44	25	45	17	26
E	19	31	9	19	20	78	46	43	54	132	41	68
G	18	23	6	9	23	31	52	51	110	111	13	48
H	13	28	11	24	76	87	30	36	11	16	23	61
X	81	107	105	118	72	104	82	97	69	105	89	57

[a] Rounded to nearest per cent.

fusoform bacteria in the mouth, but in neither instance destroyed these microorganisms to an extent such that their rapid multiplication was significantly retarded. The product did not demonstrate significant antibacterial activity for the other groups of microorganisms. Although all of the five test products used in the first experiment significantly reduced the total number of bacteria in the oral cavity, no one compound showed significant reductions for all five bacterial groups.

The second, and probably more sensitive, measure of the antiseptic activity of the mouthwashes studied is the rapidity with which the bacterial flora of the oral cavity returns to its normal count. The results of the experiments undertaken here indicate that there is no long-lasting effect to be derived from the use of the products tested.

In the *in vivo* experiments described above, the methodology was that preferred by the authors. Judging by the literature, there are almost as many suggested test methods for the evaluation of liquid oral preparations as there are interested investigators. Unfortunately, the results obtained by this variety of methods are not comparable.

It would seem almost axiomatic that if liquid oral preparations are to be evaluated, some standardized *in vivo* techniques must be adopted. It has been suggested (2) that "for a practical approach, the method of obtaining oral-cavity samples for bacteriological analysis should be associated with the intended use of the class of products under test. It should be possible to correlate the efficacy of a medicated mouthwash with an innocuous oral rinse, toothpastes with brushing alone, germicidal gums with the chewing of sterile paraffin or some similar substance, and medicated lozenges with the corresponding effect of a placebo of equal volume and weight."

If the problem of measurement of the efficacy of any oral preparation merits consideration, it should not be too difficult for some interested body of investigators to settle on a test procedure acceptable to all.

SUMMARY

Data are given here on *in vivo* studies of the antibacterial activity of several commercial mouthwashes.

It appears from these data that each of these compounds significantly reduces the total bacterial count as measured by an oral rinse test method, and that some deal selectively with given groups of microorganisms.

The data indicate, however, that while some of the compounds produced a high kill of certain groups of microorganisms, none produced a complete or even near complete destruction of any group of microorganisms. It appears also that the effect of the mouthwashes under study is of short duration.

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Paper Chromatographic Evaluation of Menthol*

By LOLA V. HOPKINS and MARTIN I. BLAKE

A method is described for the conversion of menthol to a xanthate derivative which is isolated and treated with *p*-aminobenzaldehyde which produces a red color. The strong acidity of the color reagent regenerates menthol which is determined quantitatively by a paper chromatographic procedure which is described.

THE EXTENSIVE USE of menthol in the pharmaceutical industry has led to considerable interest in analytical methods for its determination. Several chromatographic procedures have been developed. Varma, Burt, and Schwarting (1) separated menthol from menthone by a column chromatographic method. Ito, Wakamatsu, and Kacuabara (2) used a chromatostrip technique to separate menthol from Japanese mint oil. The chromatograms were sprayed with aqueous vanillin and concentrated sulfuric acid to identify the menthol spots. Hamarneh, Blake, and Miller (3) reported the separation of menthol from peppermint oil by column chromatography and the quantitative determination of menthol content by a colorimetric method.

The formation of xanthates for identification of alcohols is suggested by Guenther (4). Kariyone, Hashimoto, and Kimura (5) separated xanthates of lower molecular weight alcohols by paper chromatography.

This investigation deals with the formation of the xanthate of menthol (a secondary alcohol), and its separation from other constituents in complex mixtures by paper chromatography. The xanthate is isolated and treated with *p*-dimethylaminobenzaldehyde test solution which produces a red color that can be used to determine the menthol content quantitatively. The strong acidity of the color reagent regenerates the free alcohol from the xanthate combination; a property of xanthates noted by Feigl (6). The color reaction was first observed by Masamune (7) who determined menthol colorimetrically in tissues and blood. The reaction has been applied by Hamarneh, Blake, and Miller (3) to the estimation of menthol in peppermint oil.

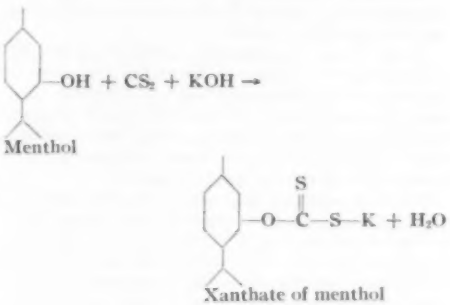
* Received November 17, 1958, from the School of Pharmacy, North Dakota Agricultural College, Fargo.
This paper is adapted from the manuscript submitted by Lola V. Hopkins which received a first prize in the 1958 Lunsford Richardson Pharmacy Awards competition.
Abstracted from a thesis submitted to the Graduate School of the North Dakota Agricultural College in partial fulfillment of the requirements for the degree of Master of Science.

EXPERIMENTAL

Apparatus and Reagents.—Aloe chromatographic chamber, 12 x 24 inches; Aloe ultra-micro pipets, 2, 4, 6, 8, 10 λ; Klett-Summerson colorimeter, with green filter No. 54 (500 to 570 mμ); light source: ultraviolet lamp, type 7420, Hanovia Chemical and Manufacturing Co., Newark, N. J.; glass-stoppered graduated cylinders, 50 ml.; Whatman No. 1 filter paper for chromatography, 4 cm. wide, in a continuous roll; menthol, racemic, U.S.P., Magnus, Mabee, and Reynard; chloroform, reagent grade, Merck; carbon disulfide, reagent grade, Merck; Drierite, W. A. Hammond Drierite Co., Xenia, Ohio; *p*-dimethylaminobenzaldehyde test solution, U.S.P. XV, prepared daily; developing solvent: toluene:*n*-propanol (1:1) prepared daily from toluene, reagent grade, Merck, and *n*-propanol, reagent grade, Fisher Scientific.

Procedure.—Ascending chromatographic technique was used. The chamber was saturated with toluene for twenty-four hours before chromatographing by placing an open beaker of toluene in the chamber. Drierite was also placed in the chamber during this period to adsorb water vapor. The trough containing the developing solvent was placed in the chamber prior to use.

The sample was prepared for chromatograming by forming a xanthate. One milliliter of the sample was transferred by pipet to a 10-ml. test tube containing 0.5 ml. carbon disulfide (delivered by pipet) and about 0.1 Gm. (two pellets) of potassium hydroxide. The test tube was shaken intermittently for exactly ten minutes. The reaction proceeded according to the following equation:



The Whatman filter paper was cut in strips 50 cm. long. The end of the strip was cut to a point. The xanthate of menthol was applied to the paper by ultra-micro pipet at a position 5 cm. from the pointed end, and equidistant from the edges of the paper. The spot was made as small as possible with a diameter not more than 0.5 cm. Ultra-micro pipets were used for spot application to facilitate the application of a definite quantity of sample to the paper.

After the spot was air-dried, the paper was

placed in the chromatographic chamber, the pointed end just dipping into the developing solvent. This promoted even distribution of the developing solvent during chromatograming. Drierite remained in the chamber during development to adsorb water vapor. Optimum development time was two hours.

In the post-development procedure, the paper was removed from the chamber and air-dried at room temperature. The xanthate of menthol was located by its yellow luminescence under ultraviolet light. A section containing the xanthate was cut from the chromatogram and placed in a 50-ml. graduated cylinder. One milliliter of chloroform and 5 ml. of *p*-dimethylaminobenzaldehyde test solution were added from a buret. The cylinder was stoppered, then shaken briefly by hand to ensure complete mixing of the contents, and set aside for two hours for optimum color development. Because of the strong acidity of the color reagent, the paper dissolved and the xanthate of menthol was converted to free menthol. The menthol and the *p*-dimethylaminobenzaldehyde test solution produced a red color which was used to determine quantitatively the menthol content.

The contents of the glass-stoppered cylinder were transferred to a Klett-Summerson colorimeter tube. A blank was prepared from a section of Whatman No. 1 filter paper treated in the same manner as the sample and equal in size to the section containing the chromatogramed sample. The scale pointer of the Klett-Summerson colorimeter was adjusted to the zero marking with the blank. The light transmittance of the chromatogramed sample was then measured.

Preparation of the Standard Curve. Racemic menthol was used in the preparation of the standard curve. Exactly 1.7500 Gm. of menthol was weighed into a 10-ml. volumetric flask, and chloroform was added to the mark. This represented a concentration of 175 mg. of menthol per ml. of solution. One milliliter of this solution was used for preparing the xanthate derivative. Since the total volume obtained in the formation of the xanthate was 1.5 ml., a dilution factor of 3:2 was involved in all calculations. Thus, after xanthate formation, the concentration of menthol was $^2/_3 \times 175$ mg. or 116.6 mg./ml. Furthermore, since λ aliquots of the xanthate were used for chromatograming, a further dilution factor was necessary. A 1-λ aliquot contained 0.117 mg. of menthol. Concentrations of menthol from 0.040 mg. to 0.933 mg. were chromatogramed by varying the concentrations of the original menthol solutions and varying the size of the λ aliquots. A standard curve was constructed by plotting scale readings *versus* menthol concentration. The concentration range of 0.120 mg. to 0.700 mg. was found to obey Beer's law.

TABLE I.—ANALYSIS OF MENTHOL SAMPLES

No.	Type Sample	Menthol Concentration, mg./ml. Taken[a]	Found[b]	Recovery, %
1	Menthol	160	159	99.37
2	Menthol	180	180	100.00
			179	99.44
3	Menthol	250	248	99.20
4	Menthol and camphor	150	154	102.66
5	Menthol and thymol	300	307	102.33
			295	98.33
6	Menthol, thymol, and camphor	150	147	98.00
7	Menthol, thymol, and camphor	550	547	99.45

[a] Concentration of original unknown solution.
[b] Average of three determinations.

Application of Method.—Menthol samples were prepared for use as unknowns and were analyzed by the proposed procedure. Menthol concentrations were also determined in the presence of thymol and camphor. The data are reported in Table I.

EXPERIMENTAL VARIABLES

***R_f* Values.**—For the toluene:*n*-propanol developing solvent, the R_f for menthol as the xanthate was 0.97. The R_f for thymol was found to be 0.02, and for camphor 0.70. Pure samples of thymol and camphor were chromatogramed without pretreatment (as in xanthate formation of the menthol) to establish R_f values. Thymol and camphor were also chromatogramed with pretreatment as in xanthate formation. The R_f values were identical. Thymol was located by dipping the developed chromatogram in half-strength iodine and potassium iodide test solution (U.S.P. XV). A brown spot appeared in about twenty minutes. Camphor was located by dipping the developed chromatogram in a 0.4% solution of 2,4-dinitrophenylhydrazine in 1.5 *N* hydrochloric acid, followed by dipping in a 10% aqueous solution of sodium hydroxide. A red-brown color developed in a few minutes. This identification procedure was a modification of the method used by Rice, Keller, and Kirchner (8). Separation of menthol from thymol and camphor was readily effected.

Developing Solvent.—After considerable experimentation, the toluene:*n*-propanol (1:1) developing solvent was found to be satisfactory. Other solvents which were examined included ether, acetic acid, chloroform, *n*-butanol, isoamyl alcohol, carbon tetrachloride, *n*-hexanol, dioxane, *o*-xylene, morpholine, benzene, water, and combinations of these.

Development Time.—Optimum development time for chromatograming was found to be two hours. For shorter periods of time, the separation of constituents was poor. Prolonged time (over three hours) gave erratic results.

Color Development.—The length of time for optimum color development of the xanthate with the color reagent was two hours. Since the color reagent is extremely sensitive to menthol, it was necessary to prepare the color reagent in a room separate from the one used for preparation of samples. Fresh color reagent was prepared daily.

Temperature.—For all determinations, the temperature during chromatograming did not vary more than 5°. It was found that temperatures above 30° produced erratic results.

Critical Volume.—Clayton (9) has recommended that the amount of developing solvent used for a specific size chamber should be reported in all chromatographic work. In this study, it was found that 40 ml. of developing solvent was optimum for the 12 x 24 inch chamber.

Number of Papers in Chamber.—The simultaneous chromatograming of more than six papers in this chamber produced erratic results.

Water Vapor.—The presence of water vapor in the chamber resulted in a spreading of the xanthate of menthol along the entire length of the chromatogram. Therefore, Drierite was placed in the chamber during the saturation period and during chromatograming.

Spot Placement.—The positioning of the spot on the chromatographic paper is critical. If it was placed too close to the end of the paper, there was some loss of the spot producing low values. If the spot covered too large an area, low results were obtained.

DISCUSSION

Menthol is commonly used in pharmacy and medicine in combination with thymol and camphor as well as in the form of peppermint oil. An assay is officially recognized in the U.S.P. XV for the determination of total menthol in peppermint oil. There is no suitable method for estimating menthol in combination with thymol and camphor.

Paper chromatography was investigated as an analytical method because of its ease and simplicity. The equipment necessary is relatively inexpensive and the reagents are common laboratory chemicals. In the first phase of the proposed method, menthol is isolated from other constituents by an ascending paper chromatographic separation.

In the second phase of the method the chromatogramed sample is treated with *p*-dimethylaminobenzaldehyde test solution. The intensity of the red color which develops is dependent upon the menthol concentration. The light transmittance is measured with a Klett-Summerson colorimeter. The menthol concentration is determined from a standard curve.

While the basic chromatographic procedure is relatively simple, caution must be used to eliminate as many variables as possible. Complete saturation of the chamber is necessary for consistent results. One of the greatest difficulties is the variation in results due to high temperatures. For accurate application of a definite quantity of sample ultramicro pipets must be used. It is especially important to remove excess sample from the outside of the pipet before applying the sample to the chromatogram.

It is important that the developing solvent be completely removed from the developed chromatogram before reacting the xanthate with the color reagent. The developing solvent reacts with the color reagent to produce high results. The use of ground-glass stoppered cylinders during the color development is necessary to prevent volatization of the reagents.

The xanthate derivative has the advantage of reducing the volatility of menthol during chromatograming yet permits the development of a color reaction with the *p*-dimethylaminobenzaldehyde test solution. The red color which develops is used for the colorimetric determination of menthol concentration. The green filter No. 54 was employed in the Klett-Summerson colorimeter after experimentation showed that the maximum absorption occurred in the 500 to 570 mμ range.

The procedure is applicable to all concentrations of menthol. When the menthol concentration is above 50%, it is recommended that the sample be diluted prior to xanthate formation in order that the chromatogramed sample fall within the range of the standard curve.

The results of this investigation suggest a simple procedure for the determination of menthol, free and in the presence of thymol and camphor. Work is in progress to apply the method to the analysis of peppermint oil.

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Neurotoxicity of Hydrogen Cyanide*

By SEYMOUR LEVINE and BENJAMIN WEINSTEIN

Instead of exposing animals to a constant concentration of hydrogen cyanide, the concentration was deliberately varied according to the condition of the animals so as to produce a steady sublethal degree of intoxication. Only in this way was it possible to produce brain lesions in a regular and reproducible manner.

THE HISTOPATHOLOGIC LESIONS induced in experimental animals by toxic vapors are important in localizing susceptible tissues or cells and in determining the mechanism of toxic action. The purpose of this investigation was to devise a technique for administration of hydrogen cyanide that would produce lesions in simple, rapid, and reproducible fashion. The problem was approached by exposing rats to constant or variable concentrations of hydrogen cyanide for various periods of time under continuous observation and sacrificing the animals, after an interval, for histopathologic study. The technique which has been developed may be applicable to the study of other noxious vapors.

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This investigation was supported wholly by Grant 184 from the National Multiple Sclerosis Society.

EXPERIMENTAL

Apparatus.—Groups of rats were exposed in a 20-liter jar. The hydrogen cyanide was generated by bubbling air through a solution of 1 to 5% potassium cyanide contained in a gas washing bottle with fritted glass disk. The hydrogen cyanide vapor was mixed with plain air in the desired proportions.

Single rats were exposed individually in 1-quart wide-mouth jars or fishbowls closed by two-hole number 14 rubber stoppers. Each stopper was fitted with an inflow Y-tube which combined the hydrogen cyanide vapor with plain air, and a straight tube for outflow (Fig. 1). Six such units were operated simultaneously with a single flowmeter and a single pump whose outflow was split into 6 parts by a glass manifold (Carrel-Dakin connecting tube). In order to permit separate control of the cyanide concentration in each unit, the air coming from each manifold port was further subdivided by a Y-tube. One arm of each Y conducted plain air directly to the corresponding jar, while the other arm led to the jar by way of a small individual fritted glass bubbler in a side-arm test tube containing potassium cyanide. Needle valve (or screw clamp) B controlled the proportion of flow in each arm, and hence the concentration of hydrogen cyanide in the jar.

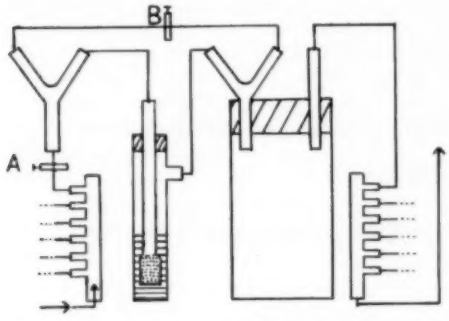

Fig. 1.—Diagram of one of the six units of the vapor exposure apparatus.

The six screw clamps A attached to the orifices of the manifold were essential for maintaining constant total flow at predetermined levels through each unit. They were partly closed so as to offer higher air resistance than the bubblers. Thus they assured a constant and equal flow through each chamber despite changes in resistance distally caused by opening and closing needle valves B during the exposure. In the absence of this arrangement the air flow from the pump would follow the path of least resistance through any of the valves B that happened to be open, rather than go through the bubblers with their inherent resistance. Before exposures, the flowmeter was temporarily removed from the pump and used to measure the outflow from each unit which was equalized by slight adjustments of screw clamps A.

Exposure to Constant Concentrations.—Adult albino rats exposed to hydrogen cyanide vapor of constant concentration became active after a few moments with increasingly violent running, jumping, and digging motions. Then there were hyperpnea, partial loss of postural tonus, convulsive twitches of extremities or tail, and occasional ineffectual running or jumping motions. The hyperpnea subsided as the rat lost its postural tonus completely and sank to the bottom of the jar. Slight movements brought on by tilting the jar soon disappeared, followed by loss of the ear twitch response to a brisk tap on the jar. Then there was progressive diminution in respiratory rate and depth until death occurred. No histopathologic lesions were detected in animals that died in this manner, presumably because there was insufficient time for histologic changes to develop.

In order to have a survival time sufficient to allow the development of lesions, lower but constant concentrations were used. If the concentration was sufficiently low, consciousness was never lost, but again, no lesions were produced even after exposures of many hours.

At intermediate concentration levels, consciousness was maintained for a while, sometimes for an hour, followed by sudden unconsciousness and death. This observation made it clear that a constant concentration of hydrogen cyanide did not mean constant experimental conditions, since the animal itself changed during such an exposure. Therefore, further attempts to produce lesions were based on keeping the rat at a constant physiological level by varying the cyanide concentration as needed.

Exposure of Groups to Varying Concentrations.—A group of eight female 250-Gm. rats received a single exposure of five to fifteen hours duration. The inflow of cyanide was reduced when many of the rats became unconscious and it was increased when some of them revived. Marked individual variation was noted so, that at any given moment, some rats were in terminal condition while others were conscious and capable of walking. Two rats died during the exposure. Of the six survivors sacrificed after forty-eight hours, 2 had brain lesions.

Ten male rats were subjected to 1 to 6 repeated exposures of five to nine hours each and 4 developed brain lesions. Five female rats were subjected to 3 to 9 similar prolonged exposures and 4 of this group developed brain lesions. In both these groups, some of the negative results were in animals who had received the maximum number of exposures.

The improved results, as compared to experiments with constant concentrations, were attributed to the nearly lethal intoxication maintained for a sufficiently prolonged time. However, it was inconvenient to conduct prolonged, multiple exposures, and there was great individual variation in susceptibility.

Exposure of Individual Rats to Varying Concentrations.—Female 250-Gm. rats were exposed in individual jars, and six units were manipulated simultaneously. The rats were kept unconscious but when there was twitching, hyperpnea, or response to tilting the jar, the cyanide was increased. When tapping the jar no longer elicited an ear twitch and when there was respiratory depression, the cyanide was decreased or discontinued. Individual variations were no longer a problem as each rat was exposed separately. A fairly even plane of sublethal intoxication was maintained for twenty to forty-five minutes. Longer exposures caused great mortality. Shorter exposures were sometimes ineffective in producing lesions. In 8 experiments involving 48 rats exposed under the optimum conditions described, 15 died during exposure and all 33 of the survivors had brain lesions.

In other experiments a somewhat milder but more prolonged and repeated intoxication was produced. Six rats were kept unconscious but with persistent muscular twitches, intermittent hyperpnea, and partial arousals for periods of two hours, repeated from 4 to 6 times. Only one rat showed a brain lesion, and this was slight.

These results indicated that a single, relatively brief exposure to hydrogen cyanide caused brain lesions in very high incidence, but only if the intoxication was maintained at a nearly lethal stage. The superiority of individual over group exposures, despite greatly shortened exposure time, was attributed to the maintenance of a constant degree of sublethal intoxication.

DISCUSSION

The optimum conditions for producing brain lesions with hydrogen cyanide were not provided by the administration of constant concentration of vapor. It was found necessary to induce a nearly lethal degree of intoxication, maintain that state for twenty minutes or more, and then permit the animal to survive long enough for lesions to develop. This could be accomplished only by varying the concentration of vapor in accordance with the condition of the animal as ascertained by constant inspection. Group exposures were unsuitable for this delicate control because of individual variations. Individual exposure in small separate jars provided a technique for producing brain lesions in high incidence (the histopathology, distribution, and pathogenesis of the lesions are discussed in another publication (1)).

These considerations may be applicable to noxious gases and vapors other than hydrogen cyanide. It is apparent that the absence of histopathologic lesions following a gas or vapor exposure may have little meaning unless the exposure was conducted under optimum conditions for the production of lesions.

SUMMARY

A technique for hydrogen cyanide administration has been described which induced a high incidence of brain lesions in rats. The rats were exposed in individual jars and maintained in a nearly lethal degree of intoxication by varying the concentration according to the condition of the animals. A simple apparatus for simultaneous operation of six individually controlled units with a single pump and flowmeter has been described.

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A Comprehensive Pharmaceutical Stability Testing Laboratory I*

Physical Layout of Laboratory and Facilities Available For Stability Testing

By LEON LACHMAN and JACK COOPER

This report illustrates the physical layout as well as the fixed equipment in a well organized stability laboratory in the pharmaceutical industry. It discusses the application of the equipment for testing pharmaceutical formulations as to their stability under varying conditions of temperature, humidity, and light intensity. Constant temperature equipment described consist of specially designed walk-in rooms as well as smaller size cabinets providing for a range of temperatures from −20 to 100°. In order to study the effect of varying humidity conditions on pharmaceutical products, constant humidity cabinets of a new design are illustrated and their operation discussed. These cabinets provide for a range of humidities from 30% relative humidity to 90% relative humidity. The influence of light on the stability of pharmaceuticals is discussed, and a specially fabricated light stability cabinet is described which simulates ordinary lighting found in the home, on a pharmacy's shelves, or in storage areas, but under exaggerated light intensity.

ALTHOUGH it has long been recognized by reliable pharmaceutical manufacturers that the potency of a drug is most important at the time it is administered to the patient, the exhaustive testing of new pharmaceutical preparations for physical and chemical stability is a procedure of relatively recent origin. Even today, not all pharmaceutical manufacturers perform extensive stability tests on new formulations. This may be due to the lack of trained personnel to plan and interpret the data resulting from such studies or to the absence of the essential equipment and comprehensive program needed to obtain pertinent and accurate stability data.

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Pharmaceutical manufacturers are becoming increasingly conscious of the absolute necessity of organizing a comprehensive stability testing program which provides adequate facilities for a skilled staff. In addition to the economic hazards involved in the introduction of an unstable product, the effects on the general scientific reputation of a manufacturer may be disastrous. With increasing competition in the pharmaceutical field, it is of considerable advantage for a new product to appear on the market as soon as possible after its formulation. However, in order to accomplish this, the manufacturer must first submit a New Drug Application to the Food and Drug Administration which, on the basis of adequate and accurate stability data, demonstrates the maintenance of labeled potency for a reasonable period of time.

The classical method usually employed to determine the stability of a new product is to expose the product to ordinary storage conditions (room temperature) for the period of time that the product would generally be stored in the normal market, e. g., anywhere from two to five years for most pharmaceuticals. An obvious disadvantage of such a procedure is the loss of time before marketing as well as the possible loss of market priority on a competitive product. Furthermore, if the preparation studied by this method appears to be unstable, there is no assurance without a similar time-consuming study that a modified product would not also be unstable.

In recent years, however, accelerated studies of stability have been employed as guides for the estimation of shelf life at ordinary shelf temperatures. Blythe (1) in 1954 made a survey of the stability testing programs of 40 pharmaceutical companies. He found that the greater number of companies use exaggerated conditions of temperature, humidity, and light to test the stability of new formulations. However, from the results presented in his report, it is clearly evident that the correlations between accelerated data to shelf storage conditions varied considerably due to different interpretations by manufacturers. This may be due to the fact that the correlations were intuitive, based on insufficient number of elevated storage conditions, or on empirical relations found in supposedly similar preparations.

Predictions of shelf life from accelerated stability studies can, however, be placed on a quantitative basis by the application of certain fundamental physicochemical principles. Studies of this type have recently been reported on in the literature (2–4), and they indicate that the shelf stability of products can be predicted within a relatively short period of time through the use of chemical kinetics. This method is applicable to simple pharmaceutical formulations as well as to more complex formulations containing several active ingredients, colorants, excipients, and the like.

It is the purpose of this report to present the design and facilities for the comprehensive stability testing program currently in use at Ciba Pharmaceutical Products, Inc. The physical plant and equipment for the Stability Laboratory used to test new formulations under exaggerated and ordinary storage conditions will be described. Through the use of this equipment and the application of certain physicochemical laws to the data obtained at the various storage conditions, it has been found that the shelf stability of new formulations can be predicted in a relatively short period of time and with a fair degree of accuracy.

PHYSICAL LAYOUT AND EQUIPMENT FOR THE STABILITY LABORATORY

The equipment for this laboratory has been chosen to allow for as diverse and varied conditions of testing pharmaceutical formulations as is feasible and necessary. With this equipment, stability tests for various formulations can readily be carried out under exaggerated conditions of temperature, humidity, and light intensity.

The range of temperature made available by the above equipment permits the testing of samples at temperatures ranging from −20 to 100°. There are three walk-in rooms set at 6°, 40°, and 50°, four laboratory size ovens set at anywhere from 60 to 100°, and one deep freeze cabinet set at −20°.

For testing formulations under varying conditions of humidity, seven cabinets are available, each one set at a different relative humidity. The range of humidities covered by these cabinets runs from 30% relative humidity to 90% relative humidity in increments of 10% relative humidity at 25°. However, although these cabinets are set at 25°, they can be changed to different temperatures and different relative humidities.

In order to determine the stability of formulations with respect to light, a special cabinet has been fabricated according to our specifications. This cabinet simulates ordinary lighting found on a pharmacy's shelves, in the home, or in various other storage areas for pharmaceuticals, but under accelerated light intensity.

This report will contain only a description of the fixed equipment for the stability laboratory. An overall drawing of the equipment and layout of the laboratory is presented in Fig. 1.

In order to present a clearer picture of the various pieces of equipment in this laboratory, it seems advisable to break down the equipment as follows, and discuss each piece of equipment in greater detail under these classifications: constant temperature equip-

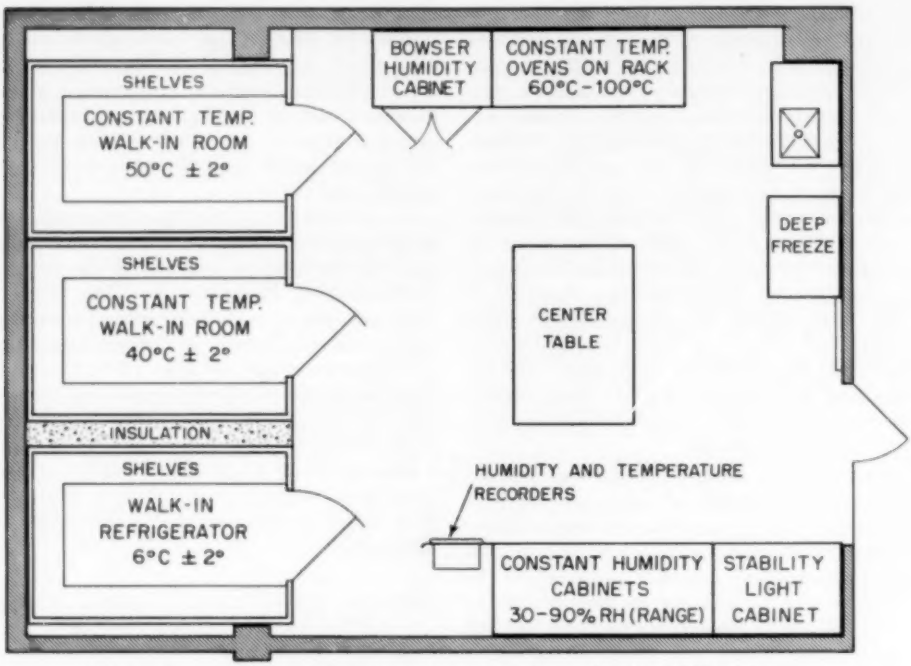

Fig. 1.—An over-all drawing of the equipment and layout for the stability laboratory.

ment, constant humidity equipment, light stability cabinet, and humidity and temperature sensing elements and recorders.

Constant Temperature Equipment.—There are three Electric Hotpack walk-in constant temperature rooms in the laboratory set at 6, 40, and 50°. A description of these rooms is presented in Fig. 2. The rooms illustrated in this figure have been manufactured according to our specifications and they have been designed to appear as one unit along one of the walls of the stability laboratory. These rooms are constructed of heavy 16 gauge aluminum throughout, and insulated with 3 inch precompressed glass wool. Glass wool was chosen as the insulating medium since it has low thermal activity and because it is nonhygroscopic, fireproof, and unharmed by corrosive fumes.

The interior dimensions of these rooms are 6 feet wide by 8 feet deep by 7 feet high. There are six tiers of 10-inch wide shelving on three sides of the room for storage of samples. These shelves are adjustable on 2-inch centers, and any or all of them can be removed if desired.

For the rooms to be kept at 40° and 50°, scientifically designed heaters and air circulation maintain a constant temperature, controlled by an adjustable mercury thermostat with one change of air every 10 minutes. The heaters consist of nichrome coils running through porcelain refractories, scientifically distributed in louvered baffles, and mounted on the three sides of the room near the floor. The air is drawn into the chamber through two ports located at the lower front section of the chamber. The air then passes over the heater banks, is heated, and rises up through the louvers and into the work chamber of the unit. After transmitting its heat to the work load, the air then passes into the ventilating duct located in the ceiling of the room. At the front of the ventilating duct is a 6-inch exhaust fan that expels the used air outside the room. The thermostat is of the sealed adjustable mercury type. It is situated

Fig. 2.—A description of the walk-in constant temperature rooms maintained at 6, 40, and 50° ± 1.5°.

on the rear wall of the room in such a position as to reflect accurately the control temperature fluctuations within the room. The accuracy of the thermostat is protected against external vibrations by a sealed mercury relay that prevents arcing in the thermostat operation.

As a safety control for temperature override, an adjustable, independent overtemperature thermal switch is built into the room to prevent the temperature of the room from exceeding the control temperature. It operates an alarm buzzer located on the front panel and the power supply to the heaters. This control can be independently set to operate from 3 to 20° above the control thermostat, and will automatically take over the control of the heaters at this higher temperature as well as automatically turn on the alarm buzzer.

For the room to be kept at 6°, there is present a refrigeration system in addition to the heating units. In this room the heated and cooled air is apportioned, resulting in a rapid, yet close control of the conditioning medium. Two separate ducts convey the heated and cooled air to a centrifugal circulating blower. These ducts are opened and closed by automatic dampers which insure the correct quantity and correct proportions of conditioned air. The streams of air coming from these two ducts are blended by the circulation blower, and then passed through a duct to the diffusers in the ceiling of the room where the conditioned air is distributed uniformly throughout the interior of the chamber.

The temperature of the three rooms is adjusted so that there is only ± 0.5° differential at the thermostat and ±1.5° between widely located points on the top and bottom shelves.

A room adjacent to the stability laboratory is employed for room temperature (ambient) storage. This room has shelving along three of its walls to accommodate samples being stored under ambient conditions.

Since samples stored at temperatures of 60° and above will generally be kept at these temperatures for shorter periods of time than at the lower temperatures, and since not all preparations which are stored at the lower temperatures will also be stored at temperatures of 60° and above, it was felt that walk-in rooms were not required for these higher temperature conditions. Instead regular laboratory size ovens appear to be of adequate capacity for these higher temperature conditions of testing.

In order to accomplish this, four Thelco convection ovens were installed in the stability laboratory for storing of samples at temperatures of 60° and above. These ovens were placed into an angle frame so that they appear as one unit and are shown in Fig. 3. These ovens set at temperatures above 60° can also be of use in accelerated short-term studies which precede the comprehensive stability study in order to screen possible formulations for the one of maximum stability.

These ovens have a thermostat sensitivity of ±0.25° and a temperature uniformity of ±1.1°. A balanced $^1/_{30}$ hp. turboblower with a system of baffles provides a smooth flow of air constantly circulated, exhibiting predetermined air flow patterns which result in a more even distribution of heated air over the entire working area.

The ovens are constructed of an aluminum interior and an enameled exterior. All sides of the oven are insulated with glass wool for better insulating qualities. The internal dimensions of the chamber are 18 inches wide by 14 inches deep by 19 inches high. Two adjustable shelves are supplied for each cabinet.

Fig. 3.—Four laboratory size ovens placed into an angle frame and set at temperatures of 60° and higher ±1.1°.

For samples stored at −20°, a Kelvinator deep freeze is employed. Its dimensions are 26 inches high by 34 inches long by 20 inches wide.

With the following constant temperature equipment just described, it is possible to test new formulations within a temperature range from −20 to 100°. From results obtained under accelerated temperature storage, one is able to predict the stability of new pharmaceuticals at ordinary shelf temperature by considering the temperature dependency of the reaction. The most satisfactory method for expressing the influence of temperature on the stability of an active constituent is the quantitative method of Arrhenius.

Constant Humidity Equipment.—In order to study the effect of varying conditions of humidity on certain pharmaceutical formulations undergoing stability testing, a six cabinet humidity installation has been fabricated for the stability laboratory by the Electric Hotpack Company. However, at the present time only four of the six cabinets are installed, but the framework for the additional two cabinets is included so that at a later date the additional two cabinets can be readily added. The overall assembly of the cabinets is presented in Fig. 4.

The relative humidities to be maintained by the four cabinets at 25° ±0.5° are 30, 60, 70, and 90% relative humidity ±3%.

All of the cabinets are constructed of stainless steel and so fabricated as to be watertight. The internal dimensions of each chamber are 19$^1/_2$ inches wide by 18 inches deep by 20 inches high.

Fig. 4.—A six cabinet constant humidity installation with four cabinets set at 30, 60, 70, and 90% ±3% relative humidity.

For the cabinets set at 60, 70, and 90% relative humidity, coolant coils, humidity nozzles, and temperature controls are required. The water for the coolant coils comes from an insulated and accurately controlled refrigerated water bath mounted on top of the frame housing the humidity cabinets. A motor driven stirrer agitates the water for precise temperature uniformity. A built-in electric pump is provided for circulating the coolant water to each chamber. The heaters in each chamber are controlled by an accurate hydraulic type thermostat. The on-off control of the heaters counteracting the constant coolant supply are intended to provide extremely close temperature control. The air in the cabinets is circulated from the front to the rear and then passes over heaters and cooling coils located behind the rear and side wall baffles. After conditioning, the air is expelled for recirculation again. Water is passed into the cabinets through humidifier nozzles in order to obtain the desired relative humidity.

For the cabinet set at 30% relative humidity, a dehumidifier is used in place of the humidifier nozzle, to cause the low relative humidity. This dehumidifier consists of a Dryomatic air dryer mounted on top of the angle frame containing the humidity cabinets. The Dryomatic unit is completely self-contained and never needs replacement of desiccant material. Otherwise, the rest of the installation is similar to that of the other three cabinets.

The heater controls for each cabinet are mounted in a recessed panel at the front of each cabinet, while the humidity is controlled by a Humidistat mounted in the chamber.

The services, the dehumidifier, and the refrigerated water bath are located on top of the angle frame containing the humidity cabinets and have been paneled off so that they are not visible.

In addition to the above humidity cabinets, a floor model Bowser humidity cabinet set at 80% relative humidity is available in the stability laboratory. This cabinet is an individual piece of equipment requiring its own services, water supply for the humidity tank, heater controls, and refrigeration. Its internal dimensions are 36 inches wide by 20 inches deep by 43 inches high. An overall view of this unit is presented in Fig. 5. Since this unit can be readily purchased and due to the fact there is sufficient manufacturer's literature available as to the temperature and humidity controls used in this unit, a discussion of same will therefore, be omitted.

Fig. 5.—A floor model humidity cabinet set at 80% ±3% relative humidity.

Light Stability Cabinet.—In order to determine the most suitable lighting system for use in observing the effect of light on a pharmaceutical formulation, a thorough investigation of the different equipment and techniques utilized for studies performed under accelerated light conditions was made. A few of the sources of light commonly employed to determine the stability of a product to light are direct sunlight, diffused light, sunlamps and other types of ultraviolet lamps, a fadeometer, and other light sources. Since sun intensity varies from season to season and day to day, and since tests are usually conducted for uniform periods of time, it is difficult to quantitate the degree of exaggeration represented by sunlight. Arny, *et al.* (5), in studying the stability of various chemicals felt that spring and fall sun, which would represent approximately the average for the year, represented 10 to 12 times the effect of north light. Furthermore, since as the intensity of the sunlight increases, the heat absorbed by the product increases, there may very well be a potentiation of the degradation due to heat besides that caused by the sunlight. In addition, since products are usually stored in a pharmacy, in a storage warehouse, or in the home, the amount of ultraviolet rays entering from the sunlight coming through the glass store

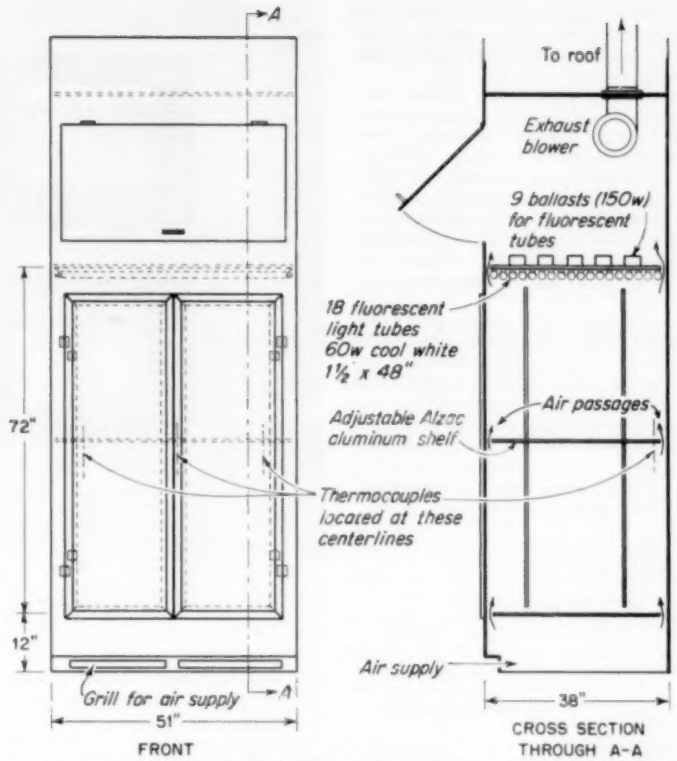

Fig. 6.—A comprehensive description of the lighting cabinet used for exaggerated light stability testing with its lighting equipment and specifications.

front or window panes of a home or a warehouse is less than 5%. Therefore, the sun ultraviolet rays would seemingly contribute very little, while light intensity should contribute significantly when studying the stability of pharmaceutical formulations with respect to light.

With the above in mind, a cabinet was designed to our specifications which contains a lighting system intended to simulate ordinary lighting found on a pharmacy's shelves, in the home, or in various other storage areas for pharmaceuticals, but under accelerated light intensity, e. g., 20 fold. The information obtained by exposing pharmaceutical formulations to this sort of accelerated lighting conditions should be extremely useful for predicting the stability of a formulation on a more quantitative basis when exposed to ordinary light conditions for an extended period of time. A later report will contain experimental data on the usefulness of this cabinet, but for the present only a detailed description of the cabinet will be given.

A comprehensive description of the lighting cabinet with its lighting equipment and specifications is presented in Fig. 6, and a photograph of the cabinet is presented in Fig. 7. It can be seen from Fig. 6 that the information contained therein is rather detailed and does not appear to require much explanation. The cabinet has been prepared according to the dimensions in Fig. 6 to cut down on light fall-off

Fig. 7.—A front view photograph of the light stability cabinet.

as much as possible. The walls were painted a flat white on the inside for reflectance purposes. The Alzac aluminum is utilized behind the lamp tubes and on the tray for light reflection purposes.

The fluorescent tubes used in this cabinet were chosen since they give off the least amount of heat per foot-candles of light intensity produced. Any heat given off by the fluorescent tubes is removed by a blower above the ballasts containing the tubes. The temperature of the shelf containing the products being tested is essentially ambient temperature, and is being constantly recorded through three thermocouple leads at the shelf attached to a Minneapolis-Honeywell 16 point Strip Chart Temperature Recorder.

The light intensity appears to be the essential factor which affects the stability of materials stored under ordinary conditions. The higher the intensity, the greater should be the effect on the material. The approximate foot-candles of intensity hitting the shelf at varying distances from the source of light can readily be calculated. For example, the approximate foot-candles of intensity available in this cabinet at a distance of three feet from the bottom can be calculated in the following manner:

$$L.\ L.^{1} = \frac{FTC \times \text{area}}{U.\ F. \times M.\ F.}$$

$$FTC = \frac{L.\ L. \times U.\ F. \times M.\ F.}{\text{area}}$$

$$FTC = \frac{3250 \text{ lumens} \times 18 \text{ lamps} \times 0.3 \times 0.75}{12 \text{ feet}} = 1100 \text{ foot-candles}$$

$$FTC = 1100 \text{ foot-candles}$$

Since the average level of light intensity in most areas where pharmaceuticals are usually stored is approximately 50 foot-candles, the lighting in the cabinet should give essentially a 20-fold increase in light intensity if the shelf containing the samples under study is three feet from the bottom of the cabinet.

Due the fact that the shelf in this cabinet is adjustable, it can be regulated to any height desired. This will enable the cabinet to be adjusted to give varying light intensities according to the following equation: Light intensity = $1/d^2$, where d = distance (feet).

Humidity and Temperature Sensing Elements and Recorders.—It appeared desirous to have a constant recording of the temperatures in the various cabinets in the stability laboratory as well as the humidities in the constant humidity cabinets in order to be able to check out the temperatures or humidities in case of irregular assay results for samples stored in these cabinets. This has been accomplished by installing two Brown Strip Chart Recording Electronik Potentiometers on a panel as shown in Fig. 8.

The strip chart recorder for the temperatures is a 16 point recorder and was attached to the various cabinets through iron constantan extension wire, 16 gauge with weatherproof over polyvinyl insulation. The thermocouples employed are manufactured by the Minneapolis-Honeywell Regulator Company. One thermocouple is inserted into each of the ports of the temperature and humidity cabinets, while three thermocouples are attached at different points on the shelf of the light stability cabinet.

Fig. 8.—Two Brown Strip Chart Recording Electronik Potentiometers used to record temperature and humidity in the several stability cabinets and rooms.

The printing record on the temperature chart consists of a (+) and a number. The number and color of printing identifies the thermocouple. The chart is graduated from 0 to 125° which is the same as the scale.

The strip chart recorder for the humidities is similar to that for the temperatures, but is only of 12 points. Here humidity sensing elements manufactured by the American Instrument Company are employed. These sensing elements are inserted into their respective humidity cabinets through ports especially prepared for them and are attached to the recorder with the aid of sensing element extension cables.

The operation of these sensing elements is based on the ability of a hygroscopic film to change their electrical resistance instantly with micro changes in moisture content (relative humidity). These elements should detect changes as small as 0.15% relative humidity and measurements are reported to be accurate within ±1.5% relative humidity.

GENERAL DISCUSSION

The foregoing presentation was intended to present to the reader an overall picture of the physical layout of the Stability Laboratory of the Pharmacy Research and Development Division of Ciba Pharmaceutical Products, Inc., as well as the fixed equipment to be employed in this laboratory. The operation of the various equipment as well as their specifications were described briefly.

[1] *L. L.* = light lumens; *FTC* = foot-candle; *M.F.* = maintenance factor; *U. F.* = utilization factor = 0.3 of level.

It appears that through the use of the facilities made available by this laboratory, it will be possible to predict the stability of new pharmaceutical formulations at ordinary shelf storage from accelerated testing data with a good degree of accuracy. This will in turn make it possible to market new pharmaceutical preparations with a definite degree of assurance as to the shelf life of the preparation and within as short a time after formulation as possible. This is becoming more and more essential due to the fact that competition in the pharmaceutical field has substantially increased within the past few years, and it is, therefore, advantageous for the pharmaceutical manufacturer to introduce on the market a new product as soon after formulation as is feasible.

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A Comprehensive Pharmaceutical Stability Testing Laboratory II*

Record and Control System Employed for the Laboratory

By LEON LACHMAN and JACK COOPER

A description of a comprehensive record and control system employed as an integral part of the stability laboratory is presented. This is intended to facilitate the recording of the numerous data that become available from the products undergoing stability testing and to provide an accurate system for sending of samples for analysis at the correct time intervals. Stability evaluation cards used to record data of a product undergoing stability testing are described. Different color cards are employed for different storage conditions. The information preprinted on the cards varies according to the type of dosage form. The labels to be placed on the products being stored are shown to be similar in color to that of the stability evaluation cards. The captions preprinted on them are discussed. A simple foolproof control method for sampling preparations at designated time intervals is demonstrated. Simplified analysis report forms to be employed when samples are sent to the other groups for testing are illustrated and discussed. In order to have available a graphic picture of the progress for the various dosage forms being tested, the use of a visual flow chart is described.

IN THE PRECEDING PAPER of this series (1), the physical layout and available equipment of a comprehensive pharmaceutical stability testing laboratory were described. The variety of dosage forms as well as the diverse storage conditions to which they are exposed render the organization and control of this laboratory a complex function. Since time is the key factor in utilizing the data obtained from the operation of such a laboratory, the system for identification of samples, recording of experimental results and planning of correct test intervals is of the utmost importance. It is the purpose of this report to describe the carefully organized, detailed but practical record and control system for stability evaluation currently employed in our Pharmacy Research and Development Division. In operation for over a year, the value of this "bookkeeping" system to the whole stability testing program has been clearly demonstrated.

Essentially, this record and control system can be divided into the following five parts: I. stability evaluation cards, II. labeling of samples under study, III. sample "withdrawal for test" file, IV. analysis report forms, and V. visual flow chart of studies in progress.

In order to obtain a clear picture of the function of each of the several parts of the record and control system, each part will now be described in detail.

RECORD AND CONTROL SYSTEM

I. Stability Evaluation Cards.—Since many formulations would be simultaneously under study, it was necessary to devise a recording system for handling the considerable volume of data in an orderly manner. A standard card 9 inches by 12 inches in size was found to be large enough to contain the required amount of information and yet of a size suitable for filing in an office file for legal documents. For each environmental condition of sample storage, a card of different color is used. Figure 1 represents the front of a representative card.

In examining the card, it can be seen that certain information is preprinted. For example, the Roman numeral in the right hand corner designates the classification of the formulation. This distinction is

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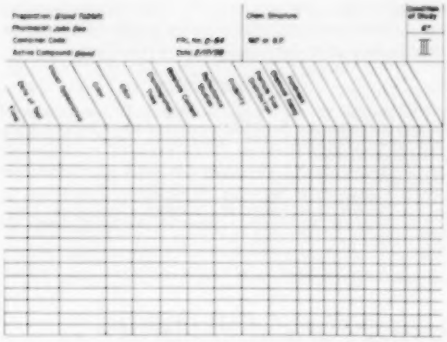

Fig. 1.—Front of a stability evaluation card.

based on certain similarities in optimal storage conditions for each classification and acts as a check on the scheduling of appropriate tests. The four classes used are:

A. Liquid Pharmaceutical Preparations (I).
B. Creams, Ointments, Jellies, and Suppositories (II).
C. Tablets, Powders, Capsules (III).
D. Lyophilized Preparations (IV).

Each classification is represented by the same color card for the same storage condition, but the preprinting of the tests to be performed will vary with the cards for each classification. However, even within a classification a particular physical or chemical test may not be applicable and should be omitted. Furthermore, there are blank spaces available for listing additional or special tests required by some formulations. Under various preprinted headings certain information concerning the active constituents will be entered prior to the actual initiation of the study.

Figure 2 shows the back of the card where provision has been made for listing the results of biological tests, the exact formula of the preparation under study and special comments of value. In scheduling a stability study, it is necessary to fill in the appropriate information on as many cards as there are storage conditions in advance of the actual initiation of the tests. These cards are then placed

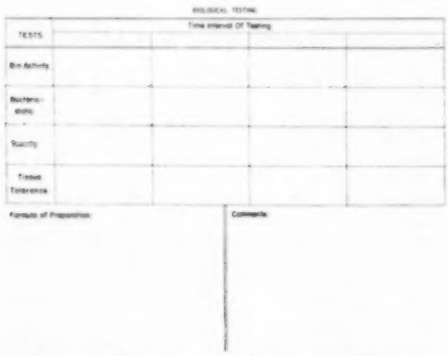

Fig. 2.—Back of a stability evaluation card.

in a folder which bears the name of the product and the folder is placed in the stability file on the day the study is officially initiated. As the study progresses, the results of the various tests are listed on the cards. A glance through the folder serves to inform a staff member as to the stability status of any product under study.

II. Labeling of Samples Under Study.—Since the labels on the samples under stability study match the color of the evaluation cards and the storage condition is preprinted in the upper right hand corner, good control of sample placement is achieved. Should a preparation intended for storage under one condition be placed in a cabinet maintaining a different condition, the label color would pointedly indicate the error. Figure 3 shows a representative label. The information on this label identifies the product, the classification, the research pharmacist, the date the study was initiated, and the pharmacist's research book number for the preparation.

III. Sample "Withdrawal for Test" File.—To avoid failure in withdrawal of samples at the appropriate testing time, a special warning file card system was devised. Figure 4 shows a sample 3 x 5 card of this type with its preprinted captions. Each drawer of the file cabinet holds the cards for one year with further subdivision into months and days.

When a stability study on a preparation is initiated, cards are prepared for each time interval of testing for the entire length of the study. It can be seen from Fig. 4 that the captions on the cards require notation of the date, the test or tests to be performed, the pharmacist's name, the name of the preparation, and the pharmacist's research book number of the preparation.

Once all the cards for the study are prepared, they are filed according to year, month, and day in the appropriate file on the day the study is officially initiated. When using this file, the technician pulls all the cards for that day and immediately begins to pull the required samples in anticipation of the tests to be performed on that day.

IV. Analysis Report Forms.—Since many of the tests for formulations under stability study are performed by divisions other than Pharmacy Research, it is necessary for these divisions to report the results obtained to the Pharmacy Research and Development Division Stability Laboratory as soon after their completion as possible. This should be done in order to keep the stability evaluation cards up to date so that the complete picture of the stability of the formulation is always available. However, in order to accomplish this, a good deal of paper work

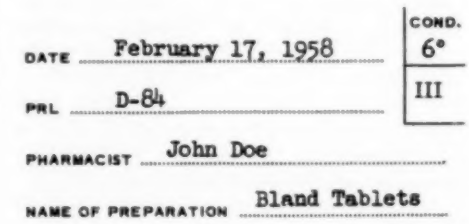

Fig. 3.—A representative label employed in the stability laboratory for samples undergoing stability testing.

DATE Feb. 17, 1958 PHARM. John Doe PRL# D-84

PREP: Bland Tablets

TESTS TO BE RUN: R.T.
40°
50°
60°
70% R.H.

Fig. 4.—"Withdrawal for test" file card.

by the cooperating divisions would be entailed if they had to write a memorandum each time they completed their tests on a formulation.

In order to substantially decrease this paper work and to facilitate reporting of data, Analysis Report Forms were prepared and a representative sample is shown in Fig. 5.

Before a sample is sent to a cooperating division for testing, these forms are filled out in triplicate and two forms are sent along with the sample to the division which will perform the tests and one copy is retained by the stability laboratory. Upon completion of the tests, the results are entered on both forms, the analyst's signature is affixed and one of the forms is returned to the stability laboratory for posting of the results on the stability evaluation cards. The completed form is filed after first removing the uncompleted form from the file. With the use of these forms, a running inventory is available with regards to analysis results still not completed by the cooperating divisions.

CIBA - Summit
Pharmacy Research and Development Division
Stability Laboratory

ANALYSIS REPORT FORM

TO: Analytical Research

SAMPLE: Bland Tablets

PRL NO.: D-84 DATE: Feb. 17, 1958

STORAGE COND.	ASSAY DESIRED	RESULTS
R.T.	chemical assay	
40°	chemical assay	
50°	chemical assay	
60°	chemical assay	

Analysis by:

Requested by: Leon Lachman

Fig. 5.—A sample of a stability laboratory analysis report form.

While on the topic of analysis by cooperating divisions, it appears advisable to mention that for a comprehensive stability testing program to be a success, the Pharmacy Research group must work in close cooperation with the Analytical and Biological groups in planning the tests for the various preparations to be studied. Usually, chemical, physicochemical, and physical assay methods can predict the stability of an active ingredient in a formulation. However, in certain instances, biological tests are more indicative of the stability of a formulation and are an important adjunct to a stability program.

V. Visual Flow Chart of Studies in Progress.—This chart is intended to give at a moment's notice the general overall status of a formulation undergoing stability study. The information made available by looking at this chart is the time interval of testing and the dates for the intervals, to what extent the study has progressed, whether a new drug application has been applied for, whether a new drug application has been approved or rejected, and at what point of the program the studies were discontinued and for what reason. The flow chart employed is manufactured by Broadmaster.[1] Through the use of different color cards which fit onto the chart, one is able to obtain a clear cut, graphic picture of the overall stability program that is in progress.

GENERAL DISCUSSION

In the previous portions of this report, the various aspects of the record and control system employed for the comprehensive stability testing program at Ciba Pharmaceutical Products, Inc., were described. It was shown how each part of the system contributes to the efficient functioning of the stability laboratory. Moreover, experience has shown this system to be an essential adjunct in the operation of this laboratory.

Through the use of the stability evaluation cards, it is possible to efficiently record all the stability data that becomes available for a new dosage form under study. By use of chemical kinetic equations and the available experimental data, it is possible to predict the stability of the product at ordinary shelf temperature by extrapolating the curve obtained from accelerated data.

Therefore, the overall effect of the equipment and the record and control system in the stability laboratory is to permit the laboratory to function efficiently as well as to make possible an orderly accumulation of sufficient and pertinent stability data. This in turn will permit the prediction of the stability of a new pharmaceutical formulation at ordinary shelf storage with a good degree of accuracy and in a relatively short period of time.

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A Folic Acid Method Involving Permanganate Oxidation*

By S. S. SCHIAFFINO, J. M. WEBB†, H. W. LOY, and O. L. KLINE

A chemical method for the determination of folic acid in pharmaceutical products is presented. The method involves digesting the folic acid in a mildly alkaline, buffered solution (3% K_2HPO_4) followed by splitting with $KMnO_4$, diazotizing the resulting PABA, and coupling with the Bratton-Marshall reagent (*n*-1-naphthyl)-ethylene-diamine dihydrochloride). In the presence of extraneous colored substances, as may be found in some pharmaceutical preparations, the chromogenic compound is extracted into isobutyl alcohol with no diminution in intensity of color, or change in wavelength of maximal absorption (550 mμ). Ferrous compounds and ascorbic acid do not interfere in the method as they are found to do in the NH_4OH digestion-zinc reduction method. The assay time with the proposed method is less because of the shorter time required to cleave folic acid by oxidation. The results are reproducible, and good folic acid recoveries are obtained.

THE PRESENT U. S. P. chemical method (1) for determining folic acid is based on the reduction of folic acid with zinc to yield *p*-aminobenzoic acid (PABA) which is diazotized and coupled with the Bratton-Marshall reagent (2) to produce a pink color which can be measured spectrophotometrically. In the application of the method to the assay of folic acid alone, or folic acid contained in pharmaceutical preparations, several difficulties have been reported. Hutchings, *et al.* (3), reported that variable results were obtained, and stated that the addition of gelatin, as a protective colloid during the zinc dust reduction, was useful in assuring a quantitative yield of the amine. Later, Kaselis, *et al.* (4), reported that this "protective" action varied with the quality of gelatin used. They recommended the use of zinc amalgam as a reductant, stating that it gave more satisfactory results than zinc dust. Glazko (5) attempted to use titanous chloride as a reducing agent. Aihara and Soto (6) reported that ascorbic acid and certain other vitamins interfere with the assay. The interference of ascorbic acid has been confirmed in this laboratory and, in addition, low results have been obtained when large amounts of ferrous salts were present in the pharmaceutical preparation being assayed.

In an effort to improve this method of assay we were attracted to the work of Allfrey, *et al.* (7), who showed that the oxidation of folic acid with $KMnO_4$ in an acid medium yields a fluorescent compound useful as a basis for a fluorometric determination of the substance. In examining this reaction, we learned that permanganate oxidation in a buffered, mildly alkaline medium (K_2HPO_4) split the folic acid to the pteridine and aromatic amine moieties in a manner comparable to the zinc reduction procedure. Also, it was found that digestion of the preparation containing folic acid was accomplished better in this buffered medium rather than in the NH_4OH medium prescribed in the present U. S. P. method.

Another refinement is the extraction of the final colored product with isobutyl alcohol in those instances where extraneous colored materials are present which interfere with the color measurement.

We have found that a method incorporating these steps eliminates uncertainties resulting from the use of various forms of reductant and saves considerable assay time since folic acid is split by the permanganate oxidation in a shorter time than by the reduction procedures. Further, ascorbic acid and ferrous salts do not interfere.

We present here in detail the method we have found satisfactory and the experimental results that are the basis for its acceptability.

METHOD

Reagents.—Potassium Phosphate Solution (Dibasic), 3%—Dissolve 60 Gm. of reagent grade anhydrous K_2HPO_4 in H_2O to make 2,000 ml.

Potassium Permanganate, 4%—Dissolve 20 Gm. of reagent grade $KMnO_4$ in H_2O to make 500 ml.

Potassium Permanganate, 0.4%—Dilute 10 ml. of the 4% $KMnO_4$ solution with H_2O to make 100 ml. Prepare fresh solution for each assay.

Sodium Nitrite, 2%—Dissolve 2 Gm. of reagent grade $NaNO_2$ in H_2O to make 100 ml.

Hydrochloric Acid, 5 *N*—Dilute 44.5 ml. of reagent grade (35–37%) HCl with H_2O to make 100 ml.

Ammonium Sulfamate, 5%—Dissolve 5 Gm. of reagent grade $NH_4SO_3NH_2$ in H_2O to make 100 ml.

N-(1-Naphthyl)-Ethylenediamine Dihydrochloride, 0.1%—Dissolve 100 mg. of reagent grade N-(1-Naphthyl)-ethylenediamine Dihydrochloride in

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H_2O to make 100 ml. Prepare fresh solution for each assay.

Sodium Chloride—Reagent grade NaCl.

Isobutyl Alcohol—Grade suitable for spectrophotometric and fluorometric use.

Folic Acid Standard Solutions.—*Stock Solution.*—Weigh accurately, in a closed system, U. S. P. Folic Acid Reference Standard, equivalent to 50–60 mg. of folic acid, that has been dried to constant weight and stored in the dark over P_2O_5 in a desiccator. Dissolve in about 50 ml. of 0.4% NH_4OH solution, and dilute with H_2O to make the folic acid concentration exactly 500 μg. per ml. Store under toluene in the dark at about 10°.

Standard Solution.—Dilute 2 ml. of the Folic Acid Stock Solution with the 3% K_2HPO_4 solution to make 100 ml. Each ml. contains 10 μg. of folic acid. Designate this as the Standard Solution. Prepare fresh Standard Solution for each assay.

Assay Solution.—Place a measured amount of the sample in a flask and add a volume of the 3% K_2HPO_4 solution equal in ml. to not less than 10 times the dry weight of the sample in Gm.; the resulting solution must contain not more than 0.1 mg. of folic acid per ml. If the sample is not readily soluble, comminute so that it may be evenly dispersed in the liquid.

Heat the mixture at 50–60°[1], agitating vigorously, and cool to room temperature. Allow any undissolved particles to settle. Filter or centrifuge if necessary. Dilute an aliquot of the clear solution with 3% K_2HPO_4 solution to a final measured volume that contains, per ml., about 5 μg. of folic acid. Designate this as the Assay Solution.

Assay Tubes.—To each of six or more tubes or reaction vessels, 40-ml. size, add 2.0 ml. of Assay Solution. Designate and prepare the tubes as follows: *Tubes A.*—To each of two or more of the tubes add 3 ml. of 3% K_2HPO_4 solution, and mix. *Tubes B.*—To each of two or more of the tubes add 1 ml. of the Standard Solution and 2 ml. of the 3% K_2HPO_4 solution, and mix. *Tubes C.*—To each of the remaining two or more tubes add 3 ml. of the 3% K_2HPO_4 solution, and mix.

To each tube designated *A* and *B* add, with mixing, 1 ml. of the 0.4% $KMnO_4$ solution and allow to stand two to three minutes. (Any appreciable change in the characteristic intense purple permanganate color during the two to three minute oxidation step denotes an excess of oxidizable material. Increase the quantity of $KMnO_4$ for assay solutions containing an excess of oxidizable material, but not more than about 1 ml. over that required to complete oxidation of foreign material.) To each tube designated *C*, add 1 ml. of H_2O and mix. (Whenever the volume of $KMnO_4$ solution is increased, add an equivalent volume of H_2O to each tube designated C so that all tubes are of the same volume.) To each tube designated *A*, *B*, and *C*, add 1 ml. of the 2% $NaNO_2$ solution; followed by 1 ml. of the 5 *N* HCl solution, and mix. Allow to stand two minutes; then to each tube add 1 ml. of the 5% $NH_4SO_3 \cdot NH_2$ solution, and mix. Swirl tubes vigorously until excess NO_2 is expelled. (Excess of NO_2 will interfere with color development.) To each tube add 1 ml. of the 0.1% *N*-(1-naphthyl)-ethylenediamine dihydrochloride solution, mix, allow to stand ten minutes for maximal color development, and proceed as directed under (*a*) or (*b*).

(*a*) *For assay solutions that contain no extraneous color in an amount that interferes with final colorimetric measurements.*—Within the next fifteen minutes (but not more than twenty-five minutes after addition of *N*-(1-naphthyl)-ethylenediamine dihydrochloride, using water as the "blank," measure the absorbance of the solution from each tube as directed under *Determination*.

(*b*) *For assay solutions that contain extraneous color in an amount that interferes with final color measurements.*—To each tube add 1 Gm. of NaCl, and 10 ml. of isobutyl alcohol, and shake vigorously two to three minutes. Centrifuge tubes until 7–9 ml. of clear supernatant isobutyl alcohol solution can be obtained from each. Within twenty-five minutes after addition of *N*-(1-naphthyl)-ethylenediamine dihydrochloride, using isobutyl alcohol as the "blank," measure the absorbance of the isobutyl alcohol solution from each tube as directed under *Determination*.

Determination.—Measure the absorbance of the solution from each tube under *A*, *B*, and *C* at 550 mμ in a suitable photoelectric colorimeter set at 0 absorbance with the "blank".

Calculation.—Calculate on basis of aliquots taken as follows, using the absorbances of *A*, *B*, and *C*: Mg. of folic acid in sample = $[(A - C)/(B - A)] \times 0.005 \times$ dilution factor.

EXPERIMENTAL

In order to standardize and test the validity of certain critical steps in the method, in some of the experiments to be described, values resulting from known amounts of folic acid were compared with values resulting from equimolar amounts of *p*-aminobenzoic acid (PABA).[2] In these instances, the folic acid samples in 5-ml. volumes were treated with the same volumes and concentrations of reagents (except where noted) and in the same order as described under *Method*. Similarly, PABA was diazotized in a volume (8 ml.) equal to that in which the folic acid samples were diazotized. All spectrophotometric measurements were made in 10-ml. volumes.

Optimal Conditions for Splitting Folic Acid by Permanganate Oxidation.—Solutions containing 10 and 20 μg. of folic acid were oxidized for three minutes with 1 ml. of each of several concentrations (from 0.2% to 4.0%) of $KMnO_4$. In addition one set of aliquots of each concentration of folic acid was oxidized with 1 ml. of 0.4% $KMnO_4$ for six minutes. Duplicate determinations were made in all cases. Following destruction of excess $KMnO_4$, diazotization of the folic acid split product and coupling with the Bratton-Marshall reagent, absorbance values of the solutions were compared with those resulting from equimolar amounts of PABA. When 1 ml. of $KMnO_4$ in concentrations up to 0.4% was the oxidant, even for periods as long as six minutes, absorbance readings of the final solutions did not differ significantly from those given by PABA solutions. With higher concentrations

[1] To avoid high blank readings, temperature during preparation of assay solution must not exceed 60°. At higher temperature diazotizable amines may be formed in the hydrolysis of organic material.

[2] The former U. S. P. Reference Standard for folic acid

of $KMnO_4$, however, absorbance readings of the treated folic acid solutions were significantly less than readings obtained from PABA solutions.

In order further to test the validity of the oxidation procedure, using 0.4% $KMnO_4$ and an oxidation time of three minutes, as previously described, absorbance values found were compared with those given by the same amounts of folic acid treated according to the zinc reduction procedure (1). The results, shown in Table I, show no significant differences in absorbance values obtained by the two procedures. Further, a comparison with absorbance values found for equimolar amounts of PABA also showed no significant differences and demonstrated a quantitative splitting of folic acid to PABA when using either method.

TABLE I.—ABSORBANCE READINGS RESULTING FROM FOLIC ACID SPLIT BY OXIDATION AND REDUCTION PROCEDURES AND FROM EQUIMOLAR AMOUNTS OF PABA

Sample No.	Absorbance[a] for 10 μg. Original Folic Acid, in 10 ml. of Final Solution: $KMnO_4$ Oxidation	Zinc Reduction	Absorbance[a] for Equivalent Amounts of PABA, in 10 ml. of Final Solution
1	0.175	0.170	0.175
2	0.180	0.175	0.171
3	0.170	0.175	0.170
4	0.175	0.175	0.180

[a] 1 cm. light path at 550 mμ.

Selection of Assay Medium.—In preliminary work on the method, experiments were performed to find a suitable medium for digestion of pharmaceutical preparations and for solution of the folic acid and, at the same time, provide a medium in which cleavage of the folic acid with $KMnO_4$ could take place quantitatively. Solutions of various concentrations of NH_4OH, $NaC_2H_3O_2$, $NaHCO_3$, and K_2HPO_4 were tried. It was not possible to obtain a quantitative recovery of folic acid when the oxidation took place in NH_4OH. With sodium acetate, the acetate ion interfered with the diazotization and coupling steps that followed. $NaHCO_3$ (5%) was satisfactory and gave about the same pH as a 3% K_2HPO_4, but the latter was finally selected because of its characteristic of precipitating ferrous ion.

Efficiency of Extracting the Chromogenic Compound with Isobutyl Alcohol.—With some pharmaceutical preparations, extraneous color was present in the final solutions which interfered with the color measurements. It was found that the chromogenic compound could be efficiently separated from foreign color by extracting it from a 10-ml. aqueous volume in the presence of 1 Gm. of NaCl, with 10 ml. of isobutyl alcohol.

Accordingly, absorbance readings of aqueous solutions of the colored compound, resulting from 10 and 20 μg. of folic acid, were compared with absorbance readings, at the same wavelength, obtained after extraction into isobutyl alcohol. The maximum absorbance for both solutions was at 550 mμ. It can be seen from Table II that the extraction took place with no diminution in the intensity of the color.

Effect of $FeSO_4$ and Ascorbic Acid on Folic Acid Assay, Using the K_2HPO_4 Digestion-$KMnO_4$ Oxidation Procedure.—Solutions containing known amounts of folic acid, and solutions of the same concentration of folic acid to which known amounts of $FeSO_4$ or ascorbic acid, and $FeSO_4$ and ascorbic acid were added, were assayed in duplicate as described under *Method*, using a new Folic Acid Reference Standard (see *Reagents*).

The solutions were as follows: Solution 1—1 mg. folic acid in 100 ml. 3% K_2HPO_4; 2—1 mg. folic acid in 100 ml. 3% K_2HPO_4 + 150 mg. ascorbic acid; 3—1 mg. folic acid in 100 ml. 3% K_2HPO_4 + 250 mg. $FeSO_4$; and 4—1 mg. folic acid in 100 ml. 3% K_2HPO_4 + 250 mg. $FeSO_4$ + 150 mg. ascorbic acid.

The amounts of $FeSO_4$ and ascorbic acid added approximated, or were greater than, amounts generally encountered in pharmaceutical preparations.

Comparison of folic acid values found for the four solutions showed no significant differences, indicating neither $FeSO_4$ nor ascorbic acid interfered in the assay.

TABLE II.—COMPARISON OF ABSORBANCE VALUES OF THE CHROMOGENIC COMPOUND IN AQUEOUS AND ISOBUTYL ALCOHOL SOLUTIONS

Solution	Absorbance[a] per 10 μg. Original Folic Acid, in 10 ml. of Solution	Absorbance[a] per 20 μg. Original Folic Acid, in 10 ml. of Solution
Aqueous (Before extraction)	0.175	0.350
	0.170	0.360
Isobutyl Alcohol (After extraction)	0.165	0.340
	0.170	0.355

[a] 1 cm. light path at 550 mμ.

In another experiment, to 1-ml. portions of four different liver extract preparations, each containing 5 mg. folic acid per ml. were added, in one instance, ascorbic acid and $FeSO_4$, and in another instance, ascorbic acid, $FeSO_4$, and an additional 5 mg. of folic acid. The solutions were assayed by the zinc reduction procedure (1) and by the K_2HPO_4 digestion-$KMnO_4$ oxidation procedure as described under *Method*, using the isobutyl alcohol extractions. Duplicate determinations were made in most cases.

The solutions were as follows: Solution *A*.—1 ml. of the liver extract; *B*.—1 ml. of the liver extract + 200 mg. ascorbic acid and 300 mg. $FeSO_4$; and *C*.—1 ml. of the liver extract + 200 mg. ascorbic acid, 300 mg. $FeSO_4$, and 5 mg. folic acid. This series of solutions were examined with each of four liver extract samples. Results obtained with the two procedures are shown in Table III.

It can be seen that, using the $KMnO_4$ oxidation procedure with K_2HPO_4 digestion, values found for Solutions *B* and *C* for the 4 samples were as expected when compared, respectively, with Solution *A*. Recoveries of added folic acid, calculated from values found for Solutions *A* and *C* of the samples, ranges from 96 to 100%. However, values obtained with the zinc reduction procedure with NH_4OH digestion were always low when ascorbic acid and $FeSO_4$ were present (Solutions *B* and *C*). In some instances absorbance readings of the controls were so high (for

TABLE III.—COMPARISON OF FOLIC ACID VALUES FOUND BY THE $KMnO_4$ OXIDATION AND BY THE ZINC REDUCTION PROCEDURES, IN THE PRESENCE AND ABSENCE OF ASCORBIC ACID AND $FeSO_4$

Sample	Average Folic Acid Found, mg.					
	$KMnO_4$ Oxidation (Using K_2HPO_4 Digestion)			Zinc Reduction (Using NH_4OH Digestion)		
	Solutions A[a]	Solutions B[a]	Solutions C[a]	Solutions A[a]	Solutions B[a]	Solutions C[a]
1	6.2	6.1	11.1	6.2	[b]	[b]
2	4.8	4.8	9.9	4.9	[b]	[b]
3	6.4	6.3	11.1	6.1	0.9	3.4
4	4.4	4.3	9.2	4.1	1.1	2.7

[a] See text for composition of Solutions. [b] Values found were negative and meaningless, see text.

example with Solutions B and C of Samples 1 and 2) that when folic acid values were calculated, they were negative and meaningless. Folic acid values for the unsupplemented Solutions A, as determined by the two methods studied, compared favorably, indicating that the liver extract solutions contained no interfering substances.

Recovery of Folic Acid Added to Pharmaceutical Preparations.—Ten pharmaceutical preparations were assayed for folic acid using the K_2HPO_4 digestion-$KMnO_4$ oxidation procedure as described under *Method*. Concurrently, the same preparations to which were added known amounts (5 or 10 mg.) of folic acid were assayed. Values for the two sets were compared respectively, and recovery of folic acid in each case was calculated. Recoveries ranged from 94 to 102%, with an average recovery of 98.7%.

Analyses of Pharmaceutical Preparations for Folic Acid Content by $KMnO_4$ Oxidation and Zinc Reduction Procedures.—In order to compare the two procedures, several preparations consisting of injectable solutions, capsules, and tablets containing $FeSO_4$, liver fractions, yeast, vitamins, and other constituents in various amounts and combinations, were assayed in duplicate and the folic acid values compared. Samples 1 to 5, according to label declarations, contained ferrous compounds and ascorbic acid, while samples 6 to 10 did not. As can be seen from Table IV, good agreement between the two methods was obtained only when these interfering substances were absent.

DISCUSSION

These experiments indicate that the permanganate oxidation procedure is applicable to a variety of pharmaceutical preparations.

The critical step in the proposed procedure is in the splitting of the folic acid. Although high concentrations of $KMnO_4$ or prolonged periods of oxidation may result in oxidation of folic acid to compounds other than the pteridine and PABA or, what is more probable, partial destruction of these primary split products, there is a satisfactory range of concentration and time that permits safe use. From reports in the literature (3, 4) and experience in this laboratory, cleavage of the folic acid molecule is also a critical step in the reduction procedures. In the method proposed, effects of excessive $KMnO_4$ oxidation are offset since the reference standard and assay solutions are exposed to the same oxidation influences. A similar approach used in the assay of riboflavin (1) has proved useful.

The interference of ferrous salts and ascorbic acid in the present U. S. P. method and the absence of their interference in the proposed oxidation procedure may be explained on the basis that these substances themselves, as present in pharmaceuticals, act as reducing agents.

In the reduction procedure ferrous ion may not be completely precipitated by NH_4OH so that it, or the ascorbic acid still present, may cause some reduction in the initial digestion step. Another possibility is that these reductants might exert an auxiliary reducing effect later when the reduction is supposedly attributed solely to zinc.

With the proposed procedure, either the ferrous ion may be more completely precipitated, or the lower pH of the K_2HPO_4 solution may inhibit reduction by it or by ascorbic acid. Furthermore, since the cleavage of folic acid is brought about by $KMnO_4$ oxidation, this oxidation may, at the same time, nullify reducing effects of the ferrous ion or ascorbic acid present.

These concepts are in agreement with our findings since, in all cases where ferrous compounds or ascorbic acid were present, low results, indicating excessive folic acid reduction, were encountered with the reduction procedure, but not with the oxidation method.

TABLE IV.—ANALYSES OF PHARMACEUTICAL PREPARATIONS FOR FOLIC ACID BY $KMnO_4$ OXIDATION AND ZINC REDUCTION METHODS

Sample No.	Folic Acid Present (Label Declaration)	Folic Acid Found, mg.			
		$KMnO_4$ Oxidation Procedure		Zinc Reduction Procedure	
		Analysis No. 1	Analysis No. 2	Analysis No. 1	Analysis No. 2
1[a]	3 mg./tablet	3.8	4.1	2.2	2.6
2[a]	3 mg./tablet	2.8	3.1	1.4	1.5
3[a]	5 mg./tablet	6.1	6.3	0.9	1.0
4[a]	5 mg./ml.	4.3	4.8	1.1	1.2
5[a]	1 mg./capsule	1.1	1.3	0.4	0.4
6	10 mg./ml.	11.3	11.8	11.5	11.9
7	1 mg./tablet	1.0	0.9	0.8	0.8
8	1 mg./ml.	1.3	1.4	1.1	1.3
9	5 mg./3 tablet	3.8	3.5	3.7	3.3
10	0.5 mg./capsule	0.6	0.6	0.6	0.7

[a] According to label declaration, these samples contained ferrous compounds and ascorbic acid.

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A Colorimetric Assay for Unoxidized Phenothiazine Derivatives: A New Complex Salt*

By JAMES A. RYAN

The lauryl sulfate salts of palladium phenothiazine derivative complexes provide a colorimetric means of following oxidative decomposition in such pharmaceutically important compounds as promazine, promethazine, and chlorpromazine. This rapid and highly specific method is well suited to routine assay of the intact drugs. The need for initial separation is eliminated in many cases.

RECENT EMPHASIS upon phenothiazine derivatives because of their importance as tranquilizing and antihistaminic agents has again focused the analytical chemist's attention on this important field. Several types of decomposition appear to take place in these compounds. One type is a mild oxidation at the sulfur atom leading to the 5-oxide or sulfoxide and eventually to the 5-dioxide or sulfone (1). Quinoid type oxidation products of phenothiazine are discussed by Granick, Michaelis, and Schubert (2–4). However, in aged or badly decomposed pharmaceutical preparations from accelerated stability studies, the extent of decomposition, or the amount of each type of decomposition product present has posed difficult problems to the analytical chemist.

The literature is replete with methods employing oxidizing agents as reagents for assaying the parent compound and its derivatives. Typical of these methods are the color reactions of Eddy and DeEds (5), Dubost and Pascal (6), and Newhoff and Auterhoff (7). The ultraviolet method of Scott (8) has been applied to measure oxidation by evaluation of the absorbance ratio of two maxima. The pure number obtained is without correlation to amount of intact material remaining in complex oxidation mixtures. The assay values obtained increase with increasing decomposition.

Failure of these methods to provide an adequate measure of intact material during stability studies on such compounds as promazine and promethazine led to an investigation of complex formation via the oxidation-labile sulfur atom as a colorimetric approach to the solution of this problem.

EXPERIMENTAL

The chlorides of gold and the group VIII transition metals were combined in dilute unbuffered solution with certain phenothiazine derivatives. The palladium complex was then selected for further investigation. Overholser and Yoe (9) used phenothiazine to determine palladium ion. These authors as well as Marcali (10) mention difficulty experienced due to the low solubility of the complex. This complex has essentially the required qualification, i. e., inability to react with the oxidized sulfur atom. Carkhuff and Boyd (11) reported a titration procedure using sodium lauryl sulfate as a titrant for quaternary and certain tertiary amines. Inasmuch as compounds such as promazine, promethazine, and chlorpromazine have the necessary molecular qualifications for forming slightly dissociated salts with the lauryl sulfate ion on the acid side, it was decided to try the lauryl sulfate ion with the phenothiazine derivative palladium complex in an effort to enhance the color formation. This resulted in the formation of a new complex salt whose visible spectrum is reproduced in Fig. 1. Note that when compared to the chloride salt the result is an approximate twofold increase in the sensitivity for promethazine accompanied by a bathochromic shift, see Equation I. Color development was poor if the lauryl sulfate ion was added prior to addition of the sample to the solution of palladium chloride, see Equation II. This suggests the complex formation involves initial formation of the palladium-phenothiazine derivative complex followed by replacement of the ionic chlorides associated with the side chain nitrogen as well as those associated with the palladium atom, palladium lauryl sulfate failing to react well with the phenothiazine derivative. A wavelength shift is also noted when the lauryl sulfate ion is added to the palladium phenothiazine complex again substantiating replacement of the ionic chlorides associated with the palladium atom, see Equation III.

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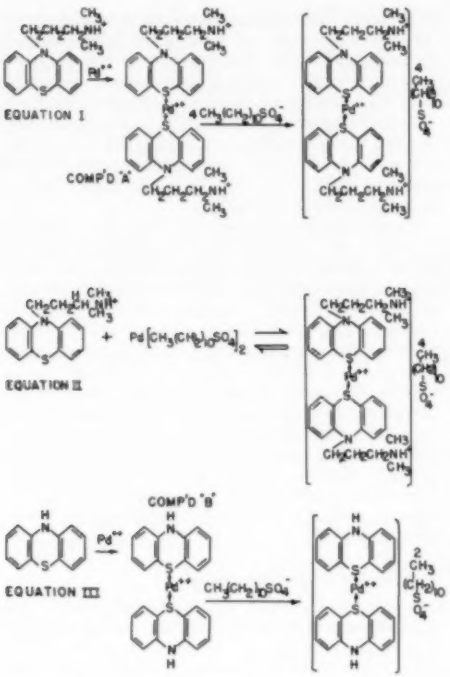

The equations are postulated for the mechanisms involved and are supported by all experimental evidence. A compound *B* above is in agreement with the elemental analysis as determined by Overholser and Yoe (9). Compound *A* was prepared (12) and complete elemental analysis confirmed a 2:1 molar ratio of promazine hydrochloride to palladium chloride. After the addition of four equivalent weights of magnesium lauryl sulfate to a solution of *A* no further change in the spectrum was noted. This experiment was carried out in a large volume of a dilute solution of *A* and the changes in the spectrum produced were followed spectrophotometrically. To support coordination involving the unoxidized sulfur atom with the palladium ion the latter was combined in dilute unbuffered solutions with the following compounds: acridine, phenoxazine, phenazine, promazine sulfoxide, and phenothiazine sulfone.

With the exception of acridine which yielded a strong greenish fluorescence and a yellow solution to transmitted light, the above compounds failed to show any visible signs of reaction.

To illustrate the applicability of this reaction to the assay of an intact phenothiazine derivative in the presence of its oxidized product, as well as a confirmation of the adherence of the assay procedure subsequently presented to the Beer-Lambert laws, a series of standard solutions of promazine hydrochloride and its sulfoxide in varying proportions of each was subjected to the assay method presented below. Results are indicated in Fig. 2.

Spectra of the Complexes

Figure 3 shows the visible spectra of promazine, chlorpromazine, and promethazine as their palladium lauryl sulfate complex salts plotted con-

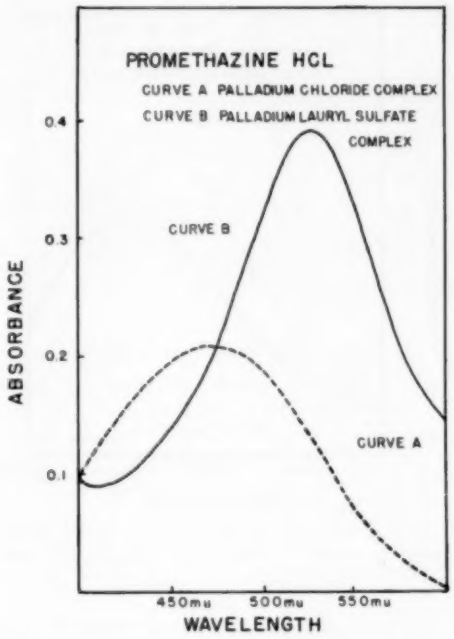

Fig. 1.—Spectra of the chloride and lauryl sulfate salts of the palladium promethazine complex.

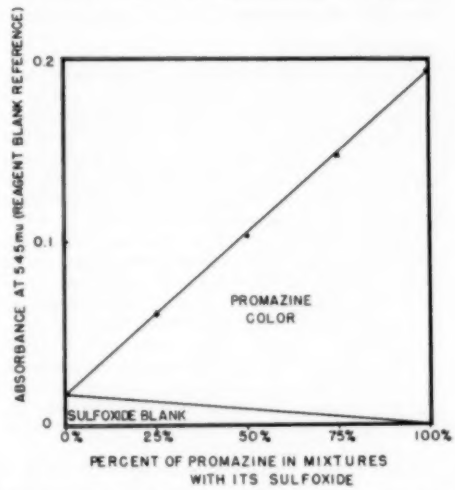

Fig. 2.—Beer's law adherence of the method in the presence of the sulfoxide.

gruently. The absorption maxima are 545 mμ, 545 mμ, and 525 mμ, respectively.

Compound *A* when compared in the infrared to its starting material revealed a number of significant spectral differences which are indicated in Fig. 4.

Conventional potassium bromide pellet technique was used in the preparation of the infrared spectra, Fig. 4. Concentrations used were 0.3 and 0.4% in potassium bromide, employing a 1-mm. path. The

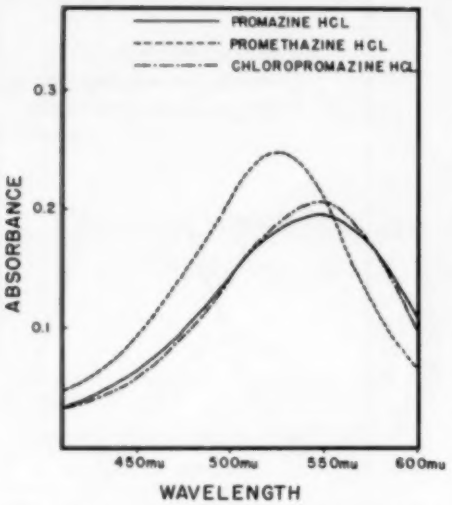

Fig. 3.—Spectra of the palladium lauryl sulfate complex salts of promazine, promethazine, and chlorpromazine.

instrument used was a Perkin Elmer Model 21. The visible spectra are reproductions of graphs as recorded on a Beckman DK-2 instrument with linear wavelength attachment. Single measurements were made on a Beckman DU spectrophotometer.

Analytical Method

Reagents.—*Stock Palladium Chloride Solution.*—Weigh 500 mg. of palladous chloride into a 250-ml. beaker, add 5 ml. of concentrated hydrochloric acid and warm the mixture on the steam bath. Add 200 ml. of hot water in small increments with continued heating until solution is complete. Transfer the solution to a 500-ml. volumetric flask and fill to the mark at room temperature with water.

Buffered Palladium Chloride Solution.—Transfer 25.0 ml. of stock palladium chloride solution to a 500-ml. volumetric flask, add 50 ml. of normal sodium-acetate solution, 48 ml. of normal hydrochloric acid, dilute to volume, and mix well.

Magnesium Lauryl Sulfate Solution.—A 0.1% aqueous solution. The magnesium salt is preferred because the solutions have less tendency to haze and precipitate. Dioctyl sodium sulfosuccinate appears to work equally well, but its salts have not been investigated in relation to this work.

Assay.—The colored test solution is prepared as follows: Transfer 3.0 ml. of the buffered palladium chloride solution to a 25-ml. Erlenmeyer flask. Add 2.00 ml. of an aqueous standard solution representing 100 to 125 μg. of the pure phenothiazine derivative for standards, or an aqueous sample solution diluted to the same level. Mix well. Add 2.0 ml. of the magnesium lauryl sulfate solution and mix well. Sample and standard solutions are prepared in duplicate. The absorbances of these solutions are determined on a suitable spectrophotometer or colorimeter, the absorption maximum of the particular complex being utilized. The instrument is set at zero absorbance using a reference solution composed of 3.0 ml. of buffered palladium chloride solution, 2.0 ml. of water, and 2.0 ml. of the magnesium lauryl sulfate solution. The concentration of the sample is then calculated. If the sample is highly colored it may be necessary to employ a sample color blank. This is prepared by omitting the buffered palladium chloride solution and substituting water in the same volume. Absorbances of all solutions are then determined using water as a reference liquid and appropriate corrections applied.

Preparation of Sample.—In most cases the only operation necessary for injections is a direct dilution to the concentration level mentioned above. In the case of tablets or capsules the following technique is usually successful. Crush a number of tablets or empty a number of capsules representative of the batch under test and grind until a homogeneous powder is obtained. Transfer an amount of the powder equivalent to approximately 10 mg. of the derivative to a glass-stoppered flask containing exactly 200 ml. of distilled water. Stopper and shake until complete solution of the active ingredient is effected. Filter through a Whatman No. 42 paper, rejecting the first 150 ml. of the filtrate. Remove a 2.00-ml. aliquot of the subsequent filtrate for the determination.

Precision and Interferences

Based upon thirteen standard solutions prepared in the manner indicated above, the average deviation of a single measurement proved to be 0.001 absorbance unit. No deviation from the arithmetical mean greater than ±0.003 absorbance unit was encountered in this series.

The following typical substances commonly found in tablets and capsules did not interfere with color development at the levels indicated after aqueous solution of 5.0 mg. of promethazine hydrochloride in their presence.

Acetophenetidin	161 mg.
Acetylsalicylic acid	226 mg.
Caffeine	32 mg.
Codeine sulfate	16 mg.
Meprobamate	200 mg.
Corn starch	60 mg.
Lactose	500 mg.
Magnesium stearate	10 mg.
Methocel 400	15 mg.
Sterotex	2 mg.

Phenol and sulfur-containing antioxidants were found not to interfere in the usual concentrations at which they appear in injections. If substances which react with palladium chloride or are incompatible with the lauryl sulfate ion are present, caution should be exercised in applying the method. High concentrations of acids, bases, salts, and organic solvents should be avoided.

If interference of any type is suspected on a given product, the test of interference should be the congruency of the visible spectrum of a complete control and a standard.

Examples of Application of the Assay to Accelerated Stability Studies

The data presented below on two selected products shows how the palladium lauryl sulfate method may be used to evaluate product stability. A choice between two different closures can be made with con-

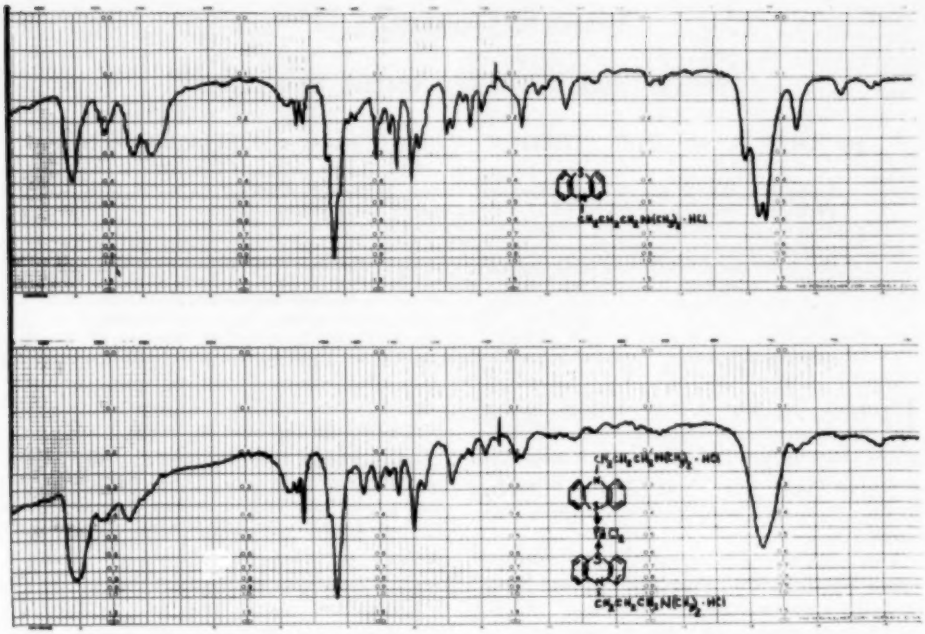

Fig. 4.—Infrared spectra of promazine hydrochloride as compared to that of its palladium complex.

fidence. The previously mentioned ultraviolet method of Scott (8) was employed below.

Example A.—Promethazine hydrochloride injection 25 mg./ml. containing a narcotic present at the same concentration level.

	Ultraviolet Method, mg./cc.	Ratio $\frac{A_{249\ m\mu}}{A_{298\ m\mu}}$	Palladium Lauryl Sulfate Method, mg./cc.
Initial	24.4	8.35	...
Six months storage under U. V. light	26.6	7.13	20.3
Six months, 45°	24.1	8.44	23.0

Example B.—Promethazine hydrochloride injection 25 mg./ml.

Initial Assay, U. V. method—26.0 mg./ml. or 104% of claim.

Storage assays by the palladium lauryl sulfate method.

Closure No. 1	Closure No. 2
25.5 mg./cc. or 102% of claim	15.3 mg./cc. or 61.3% of claim

The storage conditions of example *B* study were 45° for two years and room temperature for seven months.

SUMMARY

A method for the analysis of the more common phenothiazine medicinals has been presented. This method based on the lauryl sulfate salt of the specific palladium phenothiazine derivative complexes provides a linear measure of oxidative decomposition at the sulfur atom of these compounds.

Routine evaluation of raw material condition and product stability under accelerated storage is simplified. Choice of containers and closures is expedited.

Spectra, postulated structure, and reactions of these complexes are discussed.

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Enteric Coatings II*

Starch (and Amylose) Acetate Phthalates

By JOHN G. WAGNER, THOMAS W. BRIGNALL, and STUART LONG

Eight new whole starch (and amylose) acetate phthalates differing in acetyl and phthalyl contents were prepared by acetylation of a reactive form of starch or amylose with acetic anhydride in the presence of formamide followed by phthalylation of the partial acetates with phthalic anhydride in the presence of pyridine. Solubilities of the compounds indicated they would be useful substances for enteric coating. Tablets were enteric coated with one of the starch acetate phthalates and a commercial cellulose acetate phthalate. *In vitro*, at pH 6.9, ox bile extract but not pancreatin increased the rate of disintegration of the enteric coated tablets; hence, under these conditions, ionization of free carboxyl groups and not the action of enzymes in pancreatin is mainly responsible for the disintegration of these enteric coated tablets.

DURING the past several decades various substances and combinations of substances have been evaluated for enteric properties and a variety of artificial gastric and intestinal fluids and *in vitro* testing apparatus have been studied (1).

This is the second in the series[1] of publications which will describe the preparation and properties of some new enteric substances; discuss the factors involved in the disintegration of enteric coatings *in vitro* and *in vivo;* show that certain mathematical relationships exist which are useful in predicting the properties of enteric coatings and in comparing one type of coating with another; and correlate *in vivo* with *in vitro* disintegration times.

An enteric polymer may dissolve because of ionization of an acidic function in its molecule. For example, a cellulose acetate phthalate was reported (2) to dissolve at a pH as low as 5.7. This would be expected to cause disintegration of the enteric coating in which such a polymer was contained. However, Bauer and Masucci (3) claimed that "The disintegration of enteric coatings like cellulose acetate phthalate in the intestinal contents, which are on the acid side, is the result of the hydrolytic action of intestinal esterases." Enzymatic attack on susceptible linkages of an enteric polymer would be of value from an enteric coating viewpoint only if such an attack occurred at a faster rate than the solution of the polymer due to ionization. Resolution of the relative importance of these two possible factors would aid the search for enteric substances with improved properties.

Model enteric substances have been studied by the present authors. These substances include commercial cellulose acetate phthalate, new starch (and amylose) acetate phthalates, and two synthetic polymers which have not formerly been used in enteric coatings. Cellulose acetate phthalates and starch (and amylose) acetate phthalates have phthalate and acetate ester groups which are presumably susceptible to enzymatic attack. Startch (and amylose) acetate phthalates have (1→4)-0-α-D-glucosidic linkages presumably susceptible to attack by intestinal enzymes, whereas cellulose acetate phthalates have (1→4)-0-β-D-glucosidic linkages in the cellulose chains which are not attacked by intestinal enzymes. The synthesis of starch (and amylose) acetate phthalates and preliminary investigation of the relative importance of enzymatic attack and ionization in promoting the disintegration of enteric coatings containing a cellulose acetate phthalate or a starch acetate phthalate are given below.

Starch consists (4, 5, 6) of a mixture of a linear polymer, amylose, and a branched polymer, amylopectin. Amylose consists of anhydrous glucose units linked by (1→4)-0-α-D-glucosidic bonds. Amylopectin is composed of anhydrous glucose units linked by the same type of glucosidic bonds but with branches in the 6-position. The preparation of neither whole starch acetate phthalates nor amylose acetate phthalates has been described before.

EXPERIMENTAL

Materials.—Amylose paste, 15–20% butanol-covered (The G. Frederick Smith Chemical Co.), buffalo corn starch (Corn Products Refining Co.); formamide 99% and phthalic anhydride #2619 (Matheson Coleman & Bell, Inc.); acetic anhydride A. R. and pyridine A. R. (Mallinckrodt Chemical Works);

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[1] The first paper in this series is reference (14).

pancreatin triple strength U. S. P. (The Wilson Laboratories); and cellulose acetate phthalate type S1 (Eastman Organic Chemicals), *Anal.*—phthalyl 30.85%; acetyl 20.91%; free phthalic acid 1.75%.

SYNTHETIC METHODS

Preparation of Air-Dried Reactive Amylose.—The procedure described by other investigators (6, 7) was used. The contents of two one-ounce bottles of "15–20% amylose paste, butanol-covered" yielded 8.95 Gm. of 60-mesh air-dried reactive amylose.

Preparation of "Disintegrated Starch Granules". (*a*) *On a Laboratory Scale.*—The method of Wolff, Olds, and Hilbert (8) was utilized. Thirty-five grams of corn starch yielded 28 Gm. of "disintegrated starch granules" all of which passed through a 60-mesh screen and which contained 5.5% moisture.

(*b*) *On a Pilot Laboratory Scale.*—One kilogram of corn starch was slurried with 1,425 ml. of deionized water at room temperature and the slurry added gradually with vigorous stirring to 12,825 ml. of boiling distilled water. The paste was stirred vigorously and held at 90° for ten minutes. The temperature was raised to 100° and the paste run through a Martin-Gaulin colloid mill with a 0.002-inch setting. The slurry was cooled to 50–55° and then 14,250 ml. of denatured alcohol[2] was added slowly with vigorous stirring in such a manner as to avoid the formation of lumps of precipitated starch. The resulting suspension was run through a Fitzpatrick pulverizing machine with a 100-mesh screen. When the suspended particles had settled, the supernatant liquid was decanted; the same volume of fresh denatured alcohol was added and the suspension stirred vigorously for ten minutes. The decantation was repeated after the suspension had settled. The washing procedure was repeated twice using one-half the previous volume of denatured alcohol. The final product was isolated by filtration and dried in a vacuum oven at 80° for fifteen to eighteen hours. Yields of 650 to 725 Gm. of "disintegrated starch granules" which passed through a 60-mesh sieve and which contained about 5% moisture were obtained. Material failing to pass a 60-mesh sieve was discarded.

Acetylation.—The proportion of reagents reported by Potter and Hassid (9) was used. Acetylation in formamide was by an adaptation of the method of Jeanes and Jones (7). The modifications consisted of precooling both the formamide and pyridine to 10° ± 3° and maintaining the same temperature during the addition of the pyridine to the formamide-starch suspension; similarly the temperature was held at 10° ± 3° during addition of the acetic anhydride in increments over a fifteen-minute period (except in preparation of S. A. P. II; see Table I). After all the acetic anhydride had been added, cooling was discontinued and the mixture stirred while the temperature was allowed to rise gradually to between 22 to 28°. A further modification consisted of stopping the reactions after the variable periods of time by pouring the reaction mixture into a mixture of ice and water. Table I gives the time of acetylation from the start of the addition of the acetic anhydride to the ice treatment for each product. In each case the resulting white, fibrous precipitate was recovered by filtration, washed well by resuspension four times in deionized water, dried in a vacuum oven at 80° for fifteen hours, then ground and passed through a 20-mesh sieve. The 20-mesh powder was dried to constant weight in a vacuum oven at 80°. The acetyl contents and yields of the various products are shown in Table I.

During the acetylation of a Kg. of "disintegrated starch granules" (S. A. II, see Table I), difficulty was experienced in keeping the temperature low during the addition of the acetic anhydride in ten two-liter portions. The reaction is exothermic and in spite of the circulating brine system, the temperature rose to 22°. Additions were discontinued until the temperature was again 10°. One hour was required to complete the addition while maintaining the temperature at 10° ± 2°. The brine system was removed and the temperature allowed to rise to 25°. Stirring was continued for three hours when a clear syrupy solution was obtained. The isolation was effected as described above. The resulting starch acetate analyzed 38.4% acetyl. Sixty grams of this was phthalylated to yield S. A. P. II but the remainder of the starch acetate was partially saponified to yield starch acetate (III of lower acetyl content.

The partially acetylated starch (S. A. II) was partially saponified using the following proportions of reagents: starch acetate 14.5 Gm., acetone 207 ml., sodium hydroxide 0.5 Gm., and deionized water 1.0 ml. The starch acetate was dissolved in the acetone at reflux temperature. The sodium hydroxide was dissolved in the water and the solution added to the acetone solution of starch acetate. Heating and stirring was continued for two hours, then the reaction mixture was allowed to cool overnight. The reaction mixture was poured slowly into water and stirred for four hours. The product was isolated by centrifugation, washed well with water until neutral, and dried in a vacuum oven to constant weight at 50°. The product (S. A. III) analyzed 34.0% acetyl.

Phthalylation.—Phthalylation of the partially acetylated starch (or amylose) acetates was effected by phthalic anhydride in the presence of pyridine. The proportion of reagents reported by Malm and Fordyce (10) for the phthalylation of cellulose acetate was used. A mixture of 1 part by weight of the starch (or amylose) acetate, 4 parts of pyridine, and 2 parts of phthalic anhydride was heated to 100° with stirring and the temperature maintained for the times indicated in Table I. The reaction mixture was usually in the form of a viscous gel; it was diluted with acetone and poured into a dilute solution of hydrochloric acid containing ice. The mixture was stirred until the gel had been converted to a white or tan, fibrous solid which was recovered by filtration. The fibrous solid was washed four times by resuspension in cold water, then five times in boiling water. The product was dried in a vacuum oven at 80° for three hours, cooled, ground, and passed through a 60-mesh sieve. The powder was dried to constant weight in a vacuum oven at 80°. A summary of the products is shown in Table I.

[2] Alcohol 3A which is prepared by mixing 100 parts, by volume, ethyl alcohol and 5 parts, by volume, commercially pure methyl alcohol.

TABLE I.—SUMMARY OF DATA ON STARCH (AND AMYLOSE) ACETATE PHTHALATES

Product	Reaction Conditions: Acetylation	Reaction Conditions: Phthalylation	Analytical Values A. A. P.[c] or S. A. P.[d]: A. A.[a] or S. A.[b] Acetyl, %	Acetyl[e] %	Phthalyl,[e] %	Free Phthalic Acid, %	Moisture, %	Calculated Carboxyl (—COOH), %	Calculated Av. No. Groups/Anhydro-Glucose Residue: Acetyl	Phthalyl	Yield, %: Acetylation[f]	Phthalylation[e]
A. A. P. I.	5 hrs. 10–26°	8 hrs., 100 ± 5°, r. t. overnight	39.9 39.4	32.8	13.4	8.4	...	4.1	2.22	0.28	...	...
S. A. P. II	4 hrs.[i] 8–25°	18 hrs., 85–90°	38.4	34.7	17.4	2.25	1.34	5.3	2.67	0.39	90.0	98.9[h]
S. A. P. III	[h]	18 hrs., 100 ± 5°	34.0	31.0 30.9	19.7 19.5	3.15 2.95	1.65 1.78	5.9	2.32	0.42	85.8[g,i]	85.2[g]
S. A. P. IV	45 min., 7–22°	8 hrs., 100 ± 5°, r. t. overnight	33.3	25.3	22.2	11.7	2.0	6.7	1.86	0.46	75.4	94.1
A. A. P. V	45 min., 10–21°	8.5 hrs., 100 ± 5°, r. t. overnight	31.5	22.0	25.9	9.0	...	7.8	1.57	0.53	59.4	82.5
S. A. P. VI	40 min., 9.5–27°	20 hrs., 100 ± 5°	...	18.8	30.5	4.7	...	9.2	1.38	0.65	75.3	90.5
S. A. P. VII	45 min., 7–22°	12 hrs., 100 ± 5°	...	16.2 15.6	30.8 31.1	2.46 2.16	4.62	9.4	1.11	0.63	97.4	63.7
S. A. P. VIII	45 min., 7–22°	12 hrs., 100 ± 5°	...	23.7	32.7	3.3	...	9.9	2.01	0.80	84.8	75.7

[a] A. A., amylose acetate. [b] S. A., starch acetate. [c] A. A. P., amylose acetate phthalate. [d] S. A. P., starch acetate phthalate. [e] On phthalic acid-free, moisture-free basis. [f] Based on moisture-free starch. [g] Kilogram batch. [h] Starting material was 60 Gm. of S. A. II. [i] Overall yield inclusive of partial saponification of S. A. II to yield S. A. III. [j] Addition of acetic anhydride required one hour. [k] S. A. III obtained by partial saponification of S. A. II.

Analytical Methods.—(*a*) *Moisture.*—About 2 Gm. of starch (or amylose) acetate phthalate was weighed to the nearest mg. in a dry, tared, glass-stoppered weighing bottle and dried for at least two hours in an oven at 105–110°. The sample was cooled and weighed.

(*b*) *Acetyl in Starch (or Amylose) Acetates.*—The pyridine method of Murray, Staud, and Gray (11) was used.

(*c*) *Free Phthalic Acid.*—The ether extraction and titration method of Malm, Genung, and Kuchmy (12) was used.

(*d*) *Phthalyl.*—The titration method of Malm, Genung, and Kuchmy (12) was used. The ether extracted material from the above determination was freed from ether in a desiccator and dried for two hours at 100–110°. The solvent was a mixture of 1:1, by volume, pyridine and acetone. Blanks were run on the solvent.

(*e*) *Acetyl in Starch (or Amylose) Acetate Phthalates.*—The apparent acetyl (total alkali consumption calculated to acetyl, equivalent weight 43) was determined by the saponification-in-solution method of Malm, Genung, Williams, and Pile (13) using a solvent mixture of 1:1, by volume, pyridine and acetone. Analyses were performed on phthalic acid free samples as above. The actual acetyl value was calculated by means of the equation:

$$\% \text{ acetyl} = \% \text{ apparent} - \left(\% \text{ phthalyl} \times \frac{86}{149}\right).$$

(*f*) *Carboxyl.*—Calculated from: % free carboxyl = $45/149 \times$ % phthalyl.

SOLUBILITIES

All starch (and amylose) acetate phthalates prepared were readily soluble in 5% aqueous sodium hydroxide, 10% aqueous ammonia, acetone, methyl ethyl ketone, and mixtures of acetone or methyl ethyl ketone and alcohol, although alcohol itself was a nonsolvent. At least 10% w/v solutions in the organic solvents could be prepared.

All starch (and amylose) acetate phthalates prepared were soluble in 5% aqueous sodium bicarbonate with the exception of A. A. P. I which had the lowest per cent carboxyl of the group. Similarly all products except A. A. P. I were readily soluble in the artificial intestinal fluids, pH 6.9 (see Table III). All products were completely insoluble in simulated gastric fluid U. S. P. XV.

The solubility of some of the products in phosphate buffers was determined. Ten milligrams of compound and 50 ml. of buffer were placed in a stoppered bottle. The bottles were attached to a wheel which rotated six revolutions per minute in a constant temperature bath held at 40°. The bottles were examined after one hour and after four hours. Phosphate buffers differing in pH by 0.05 pH unit were tried for each compound until that compound was just completely soluble after one hour and after four hours. Table II lists the per cent carboxyl in

the compound and the minimum pH of the buffer in which it was soluble after four hours. In general, complete solution after one hour required a buffer from 0.05 to 0.20 pH unit higher than that given for four hours. The 3 products with 6.7, 7.8, and 9.2% free carboxyl content dissolved at a lower pH than the cellulose acetate phthalate with 35.2% phthalyl and 10.6% carboxyl reported by Malm, Emerson, and Hiatt (2).

TABLE II.—RELATIONSHIP BETWEEN THE PER CENT CARBOXYL IN THE STARCH (OR AMYLOSE) ACETATE PHTHALATE AND THE pH AT WHICH IT DISSOLVED

Per Cent Carboxyl	Minimum pH of Phosphate Buffer[a]
5.3	6.30
5.9	6.00
6.7	5.60
7.8	5.55
9.2	5.55

[a] Minimum pH of phosphate buffer at which 10 mg. of polymer just completely dissolved in 50 ml. of buffer at 40° after four hours.

COATING OF TABLETS

Compressed tablets containing 3 gr. of barium sulfate U. S. P. and lactose as diluent were prepared from a starch-syrup granulation. One lot was subcoated with a gelatin-syrup solution using a mixture of precipitated calcium carbonate, talc, and acacia powder as dusting powder. This lot was enteric coated with cellulose acetate phthalate by applying to the tablets a 10% w/v solution of the polymer in a mixture of acetone and alcohol. This lot of enteric coated tablets were those designated lot III in Table III of the previous publication (14). The other lot of compressed tablets was subcoated with a sodium carboxymethylcellulose-sugar-water solution (15) using the same dusting powder. This lot was enteric coated with starch acetate phthalate by applying to the tablets a 10% w/v solution of the polymer in a mixture of methyl ethyl ketone and alcohol. This lot of enteric coated tablets were those designated lot IV in Table III of the previous publication (14). U. S. P. supreme talc was used as dusting powder during the enteric coating of both lots of tablets.

The average weights of enteric polymer and dusting powder applied per tablet and the average volumes, weights, and thicknesses of the enteric coatings with their standard errors are given in the previous publication (14).

DISINTEGRATION TESTS

Artificial Gastric Juice, pH 1.2 and Water.—The *in vitro* resistance of the enteric coatings to simulated gastric fluid, U. S. P. XV and to water was reported in the previous publication (14).

Artificial Intestinal Fluids, pH 6.9.—The buffers shown in Table III were utilized.

The disintegration apparatus was that described in the U. S. P. (16). The temperature of the buffers were maintained at 37° ± 2°. Each run included six tablets in one basket and two runs were carried out on each lot in each buffer. The runs and samples were completely randomized. The end point for each tablet was taken when the tablet was completely disintegrated and 99 to 100% of the entire disintegrated tablet and coating had passed through the screen of the disintegration apparatus. The results of the disintegration tests in the four buffers are shown in Table IV. It was found that when the enteric coated tablets were run two hours in simulated gastric fluid U. S. P. XV then transferred to buffer A that the average values of disintegration time in buffer A were approximately the same as those in Table IV under buffer A where the enteric coated tablets were put directly into buffer A.

Comparison of Spread and Errors.—The average disintegration times of the enteric coated tablets in buffers A, B, C, and D were 45.0, 44.5, 55.0, and 55.0 minutes, respectively. The average disintegration times of the enteric coatings (obtained by subtraction of the average disintegration time of the subcoated tablets from the average disintegration time of the enteric coated tablets) in buffers A, B, C, and D were 38.8, 38.3, 47.8, and 46.7 minutes, respectively. Numerically the average disintegration time of the enteric coated tablets is 23% greater in buffers C and D which contain no ox bile extract than in buffers A and B which contain ox bile. Numerically the average disintegration time of the enteric coated tablets is about 0.5% higher in buffers A and C which contain pancreatin than in buffers B and D which do not contain pancreatin.

Barlett's test (17) was used to test the homogeneity of the variance of disintegration time in the four different artifical intestinal fluids. The test indicated some lack of homogeneity. However, despite the high χ^2 value an analysis of variance seems justified (18, 19). Based on a large number of similar disintegration tests run in this laboratory there is little evidence of a relationship between standard deviation and average disintegration time although such a relation is usually to be expected with time measurements. Occasional erratic differences in variances appears to be due mainly to nonuniformity in the coatings arising from the coating process. Some improvement in homogeneity was obtained by using log time; however, χ^2 was still significant.

Analysis of Variance.—Using the "time (T)" values directly, an analysis of variance of the data presented for the enteric coated tablets, lots III and IV in Table IV was made. The results of the analysis are shown in Table V.

The conclusions which may be drawn from the analysis of variance are as follows: (*a*) The pooled disintegration times of the tablets enteric coated with cellulose acetate phthalate and starch acetate phthalate in buffers containing pancreatin (buffers A and C) do not differ significantly from the pooled disintegration times of the same tablets in buffers which no not contain pancreatin (buffers B and D). These data indicate that the enzymes contained in pancreatin U. S. P. do not aid in the dissolution of these enteric coatings at pH 6.9. (*b*) The pooled disintegration times of the tablets in buffers containing ox bile extract (buffers A and B) are significantly lower than the pooled disintegration times of the tablets in buffers which do not contain ox bile (buffers C and D). (*c*) There is no significant difference in buffers A and B with respect to the total of

TABLE III.—ARTIFICIAL INTESTINAL FLUIDS, pH 6.9

Ingredient	Buffer A	Buffer B	Buffer C	Buffer D
Pancreatin U. S. P.	10.0 Gm.	...	10.0 Gm.	...
Ox bile extract U. S. P.	4.0 Gm.	4.0 Gm.	...	...
M/5 Potassium biphosphate	250.0 ml.	250.0 ml.	250.0 ml.	250.0 ml.
M/5 Sodium hydroxide	140.0 ml. (approx.)	140.0 ml. (approx.)	140.0 ml. (approx.)	140.0 ml. (approx.)
Deionized water *q. s.* to	1,000.0 ml.	1,000.0 ml.	1,000.0 ml.	1,000.0 ml.
pH	6.9	6.9	6.9	6.9

TABLE IV.—DISINTEGRATION TIME OF TABLETS IN ARTIFICIAL INTESTINAL FLUIDS, pH 6.9

Lot		Buffer A	Buffer B	Buffer C	Buffer D
		Average Disintegration Time ± Standard Deviation, (min.)			
III (enteric coated)	Run 1	42.7 ± 2.1	43.8 ± 4.8	63.2 ± 4.0	59.0 ± 4.9
	Run 2	49.1 ± 2.0	42.3 ± 2.9	56.8 ± 2.3	55.1 ± 5.6
IV (enteric coated)	Run 1	42.8 ± 7.9	45.8 ± 2.1	48.7 ± 4.2	49.8 ± 6.9
	Run 2	45.4 ± 1.9	46.3 ± 3.8	51.3 ± 3.9	56.1 ± 2.9
III (subcoated)	Run 1	8.2 ± 0.8	8.3 ± 0.7	9.7 ± 0.8	13.8 ± 0.4
	Run 2	8.6 ± 0.8	8.3 ± 1.2	9.9 ± 1.4	10.7 ± 1.8
IV (subcoated)	Run 1	4.2 ± 0.2	4.4 ± 0.2	4.5 ± 0.1	4.7 ± 0.5
	Run 2	4.0 ± 0.3	4.0 ± 0.3	4.8 ± 0.9	4.0 ± 0.9

TABLE V.—ANALYSIS OF VARIANCE OF T VALUES OF ENTERIC COATED TABLETS III AND IV

Source of Variation	d. f.	M. s.	F Test
Total	95	...	.....
Among lots	1	248.7	$F = \frac{248.7}{174.1} = 1.43$
Among buffers			
(A + C) − (B + D)	1	1.4	F = <1
(C + D) − (A + B)	1	2,516.4	$F = \frac{2516.4}{174.1} = 14.46^a$
A − B	1	2.8	F = <1
Interaction (buffers × lots)	3	174.1	$F = \frac{174.1}{57.1} = 3.05$
Between duplicate runs	8	57.1	$F = \frac{57.1}{17.4} = 3.28^b$
Within runs	80	17.4	

[a] Significant at 0.05 level. [b] Significant at 0.01 level.

the disintegration times of the enteric coated tablets in the buffers. (d) The variation in disintegration times between runs of six tablets is significantly greater than the variation within runs. This has been observed with larger groups of enteric coated tablets. (e) There is no significant difference between lots with respect to disintegration times in the four buffers.

DISCUSSION

It is interesting to compare the standard deviations for runs of six tablets in the various buffers used in this study with those reported in the literature or calculated from data in the literature. Crisafio, Taylor, and Chatten (19) reported a very wide range of standard deviations calculated from the disintegration times of commercial enteric coated tablets in artificial intestinal fluids, pH 7.5 and 8.0, containing pancreatin but not containing ox bile. Brindamour and DeKay (20) reported disintegration data for a cellulose acetate phthalate-coated tablet and a commercial enteric coated tablet in various artificial gastric and intestinal fluids. From their data standard deviations of six and twenty-three minutes were calculated for the cellulose acetate phthalate-coated and the commercial enteric-coated tablets, respectively, in their number 10 fluid[3] and standard deviations of seven and ten minutes were calculated for the cellulose acetate phthalate-coated and the commercial enteric-coated tablets, respectively, in their number 12 fluid.[4] The average of the standard deviations (based on 6 tablets and itemized in Table IV) for the enteric coated tablets in buffers A, B, C, and D are 3.5, 3.4, 3.6, and 5.1 minutes, respectively.

Both of these literature sources indicate that lowering the pH of the artificial intestinal fluids increases the range and hence the standard deviations of the disintegration times of enteric coated tablets. Similar to the results reported in this paper Brindamour and DeKay (20) reported that the presence of

[3] Similar to buffer A in this report but their number 10 fluid contained calcium chloride and had a pH of 6.7.
[4] The same as buffer A in this report except that their number 12 fluid contained calcium chloride.

ox bile in the intestinal solutions caused the tablets to disintegrate more quickly; their data also indicate that pancreatin had no similar effect. In reviewing the literature on bile salts Haslewood (21) points out that recent work indicates bile salts form micelles in a way not fundamentally different from that well known in the case of detergents. Solubilization of fats, etc., in bile salt solutions may be explained on the theory of "solubilization" which has been applied to explain similar properties of detergents generally. The lowering of surface tension and the detergent action exerted by bile salts probably explains the action of ox bile extract in increasing the disintegration rate of enteric coated tablets.

SUMMARY AND CONCLUSIONS

1. Six new whole starch acetate phthalates and two new amylose acetate phthalates were synthesized.

2. Solubilities of these two compounds indicate that all but one of them would be useful substances for enteric coating. It was shown that the minimum pH at which the compounds dissolve is dependent upon the per cent free carboxyl content of the polymers.

3. Subcoated tablets were satisfactorily enteric coated with a starch acetate phthalate and with a commercial cellulose acetate phthalate.

4. Initial disintegration tests indicated that the tablets enteric coated with starch acetate phthalate did not have as high a resistance to artificial gastric juice, pH 1.2, as tablets enteric coated with cellulose acetate phthalate. There was no significant difference between the two lots of enteric coated tablets with respect to initial disintegration times in four artificial pancreatic fluids, pH 6.9.

5. It was shown that at pH 6.9 pancreatin U. S. P. had no influence on the disintegration rate of tablets enteric coated with starch acetate phthalate or cellulose acetate phthalate. It was shown that at pH 6.9 ox bile extract significantly increased the rate of disintegration of these enteric coated tablets. These data indicate that *in vitro* at pH 6.9 ionization of free carboxyl groups in these polymers is mainly responsible for the solution of the polymers and the disintegration of the enteric coatings.

6. The hypothesis is presented that the detergent action of bile salts in ox bile extract is responsible for the increased rate of disintegration of these coatings in the presence of ox bile extract.

7. Both lots of enteric coated tablets disintegrated completely in a phosphate buffer, pH 6.9, containing neither pancreatin nor ox bile extract.

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A Chromatographic Technique for the Identification of Fluorescein and Phenolphthalein Derivatives*

BY C. ROBERT McMILLION and H. A. BROWN DUNNING, Jr.

New and simplified procedures are described for the chromatographic identification and separation of a number of fluorescein and phenolphthalein derivatives. The procedures employ phosphate buffers as a mobile phase. Methods are described for paper strip and cellulose column chromatography.

IN THE SYNTHESIS of fluorescein and phenolphthalein derivatives a number of by-products are formed. These products are difficult to separate by conventional means. As a result they are usually present to some extent in the final product. These compounds are weak acids and, as such, they are usually soluble in organic solvents and only slightly soluble in water. The salts of the same compounds have the opposite solubilities. This characteristic makes them unsuitable for general chromatographic

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techniques based on two phase systems. Partition methods can be used, however, as Lederer (1) and Graichen (2) have described identification techniques for phenolphthaleins and fluoresceins, respectively.

Experience in this laboratory has shown that partition methods tend to form long overlapping zones. In addition, a considerable amount of work and time is required for preparing the mobile phase. Some systems are not stable over a long period of time due to evaporation or esterification and are very sensitive to temperature changes.

The procedure described here is efficient and very simple. The solvents used are phosphate buffers. The advantages of this procedure are: (*a*) A variety of phosphate buffers may be kept for long periods of time if a few crystals of thymol are added to prevent the growth of microorganisms; (*b*) it doesn't require changing the atmosphere in the chromatography jar when a change in buffer system is desired; (*c*) the materials used are all inexpensive; (*d*) well defined zones are usually formed; (*e*) the identification can be completed in two hours; (*f*) variation between runs is very slight if equilibrating time and conditions are kept constant; (*g*) the same solvents can be used with cellulose columns to separate larger quantities; and (*h*) small temperature changes have a negligible effect on the results.

EXPERIMENTAL

Materials

Whatman No. 1 paper ($1^1/_2$ inches wide). Reagent grade Na_3PO_4. Reagent grade NaH_2PO_4. Chromatography jar 12 × 24 (descending type) with a stoppered hole in plate glass top. Micro-pipets (10 λ size). Whatman cellulose powder (ashless, standard grade). 14 × 800 mm. glass column. Automatic fraction collector (drop counting type).

Paper Chromatography

Preparation of Mobile Phase.—Two parent phosphate solutions were prepared—0.5 *M*[1] Na_3PO_4, and 0.5 *M* NaH_2PO_4. A portion of the Na_3PO_4 solution was placed in a beaker equipped with pH electrodes and a mechanical stirrer. The NaH_2PO_4 solution was added until the desired pH was reached. Several of the fluoresceins were separated using a 0.5 *M* solution of NaH_2PO_4 (reagent grade) as a mobile phase.

Preparation of Sample.—The samples were prepared as 1% solutions in 0.5 *M* Na_3PO_4.

Preparation of Chromatography Jar.—The type of jar used was of the descending type 12 × 24 inches. The plate-glass top was equipped with a stoppered hole such that the buffer could be added with a minimum of exposure. Two inches of water was kept in the bottom of the jar. Absorbent cotton or other wicking material was not used because such conditions would be difficult to reproduce.

Procedure.—Two λ (0.002 ml.) of the 1% sample solution was added to the paper strips. An attempt was made to keep the spots uniformly small. After the spots had dried the strips were placed in the jar. The top was replaced rapidly and they were allowed to equilibrate for one-half hour before the buffer was added. The strips were removed two hours later and allowed to air dry. They were then sprayed with a dilute solution of NaOH in 50% alcohol. The spots were then viewed in visible and ultraviolet light. The R_f values were determined for the point of greatest intensity.

Cellulose Column

Preparation of Column.—A chromatography column 14 × 800 mm. was used. It was equipped with a stopcock and a fritted glass disk as a cellulose support. A slurry of cellulose powder and water was poured into the column with 5 pounds of air pressure being applied after each of the small additions. A water level was maintained such that the slurry being added did not disturb the top of the column. After packing to a height of 700 mm. it was washed carefully with 0.5 *M* phosphate buffer. A buffer of pH 11.0 was used for phthaleins and pH 8.0 buffer was used for fluoresceins.

Operation of Column.—The sample to be chromatographed was prepared as a 0.5% solution in 0.5 *M* Na_3PO_4. On the top of the cellulose 0.02 cc. of the sample was placed. The chosen buffer was then added carefully such that the sample was washed into the column. Hydrostatic pressure was used

TABLE I.—R_f VALUES FOUND USING 0.5 *M* PHOSPHATE BUFFERS

Compound	pH 8.5	pH 11.0
Tetraiodofluorescein	0.01	..
Tetrabromofluorescein	0.05	..
Diiodofluorescein	0.11	..
Dibromofluorescein	0.12	..
Unsym. Dibromofluorescein	0.15	..
Diiodotetrachlorofluorescein	0.16	..
Nitrofluorescein[a]	0.25, 0.35	..
Fluorescein	0.41	..
Tetrabromophenoltetrabromophthalein	..	0.18
o-Phenylphenolphthalein	..	0.22
Dibromophenoltetrabromophthalein	..	0.34
Tetraiodophenoltetrachlorophthalein	..	0.35
Phenoltetrabromophthalein	..	0.43
Tetrabromophenoltetrachlorophthalein	..	0.45
Diiodophenoltetrachlorophthalein	..	0.48
Dibromo-*o*-phenylphenolphthalein	..	0.50
Dibromophenoltetrachlorophthalein	..	0.56
Phenoldibromophthalein	..	0.63
o-Cresoltetrachlorophthalein	..	0.65
Phenolphthalein	..	0.65

[1] All molar solutions are in terms of sodium ion concentration.

[a] Phenol condensed with 4-nitro phthalic acid yields two isomers.

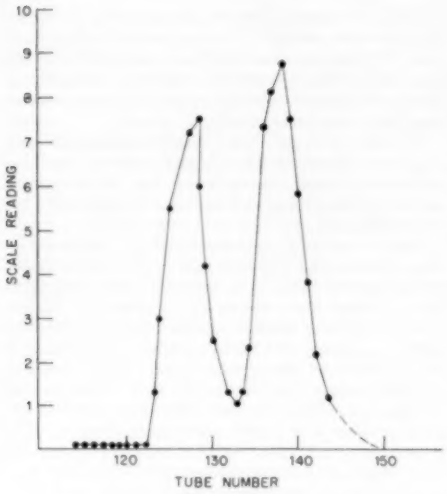

Figure 1.

to maintain a 40-cc. per hour rate of flow. An automatic fraction collector was used to collect 10 drops per tube.

Evaluation of Effluent.—Five cubic centimeters of distilled water was added to each tube and the optical density at 525 mμ determined on a Fisher Electrophotometer.

DISCUSSION

The R_f values given in Table I are approximate since no attempt was made to attain complete equilibration or to maintain a constant temperature. Results are easily reproduced, however, if the paper strips are equilibrated uniformly each time prior to adding buffer to the troughs. Several experiments were run on the fluoresceins using a wide range of buffers. This had little or no effect since the same R_f values were obtained at pH's of 8.0, 8.5, and 9.0. The effect of using more dilute buffers (0.05 → 0.005 *M*) was to cause tailing of the spots. The results obtained using 1.0, 0.5, and 0.1 *M* buffers were practically identical.

A cellulose column was used to separate the two isomeric nitrofluoresceins obtained by condensing 4-nitrophthalic acid and resorcinol. The buffer used was 0.5 *M* phosphate at pH 8.0. A procedure has been described for the separation of the aminofluorescein isomers, using *n*-butanol and cyclohexone with a column of buffered Hyflo-Supercel (3).

Figure 1 shows the results of applying a known solution of phenoltetrabromophthalein and phenoldibromophthalein to a cellulose column, using the procedure outlined in the experimental section.

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Some *in Vitro* Effects of Chlorpromazine, Lysergic Acid Diethylamide, and 5-Hydroxytryptamine on the Respiration of Rat Brain*

By WESLEY C. STARBUCK and HAROLD C. HEIM

Chlorpromazine, *in vitro*, produced a marked inhibition of oxygen uptake of brain homogenates while serotonin elicited only a slight inhibition. Lysergic acid diethylamide did not alter respiration nor did this compound evoke any effect on the inhibition caused by chlorpromazine or serotonin. It was found that the intraperitoneal administration of chlorpromazine did not alter brain serotonin levels and that brain from the treated animals respired at the same rate as that from untreated animals.

MANY years ago it was shown that certain drugs produce effects on oxidative processes of the brain, *in vitro* (1). Extension of this work by numerous investigators has led to the hypothesis that the mechanism of action of drugs which exert effects on the brain can, at least in part, be explained in terms of the effects produced by the drugs on enzymatic processes *in vivo*. In 1943 (2) it was observed that lysergic acid diethylamide (LSD) produced hallucinogenic effects. This compound represents only one of a series of drugs which have been known, for centuries, to produce mental aberrations following administration. The isolation of 5-hydroxytryptamine (5-HT) from brain (3) led to the suggestion that this compound might be, in some way, involved in brain function (4).

It has been postulated that mental aberrations induced by LSD might be due to a metabolic antagonism between LSD and 5-HT (5). It has been shown that the administration of re-

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serpine is followed by a release of 5-HT in brain (6) and that the effects elicited on the central nervous system by either 5-HT or reserpine are blocked by LSD (7). Several investigators have reported that the administration of chlorpromazine does not cause the release of 5-HT (8). It has been reported that chlorpromazine has a potentiating effect upon the narcosis produced by barbiturates and by ethanol, and this same potentiating effect has been observed with 5-HT (9, 10). The same authors report that LSD antagonizes the potentiating effects of 5-HT on narcosis induced by barbiturates. It also has been found that LSD antagonizes the potentiating effect produced by chlorpromazine upon barbiturate-induced narcosis (10). Some studies have revealed that chlorpromazine inhibits oxygen uptake by brain, *in vitro* (11, 12), and the suggestion has been made that the barbiturates may elicit their effects on the brain by interfering with cellular metabolic processes (13).

Because the mechanisms by which 5-HT, LSD, and chlorpromazine produce their effects are not well understood, it was decided to study the effects of these drugs, singly and in combination, upon the respiration of brain homogenates.

EXPERIMENTAL

Adult Sprague-Dawley rats of either sex and weighing approximately 250 Gm. were used as the experimental animals in this study. Each animal was killed by decapitation after which the brain was immediately removed, washed, blotted dry, and weighed. The brain was then transferred to a glass homogenizer immersed in an ice bath and containing sufficient 0.05 *M* phosphate buffer, pH 7.4, so that 0.3 ml. of homogenate contained 50 mg. fresh tissue.

The substrate was a solution containing a mixture of pyruvate and fumarate prepared by dissolving the required amount of fumaric acid in sufficient 10 *N* NaOH to effect solution after which 50 ml. of distilled water was added. Sufficient sodium pyruvate was then added and the pH of the solution was adjusted to 7.4 with dilute HCl. The volume of the solution was then made to 100 ml. with distilled water. The substrate and reagents used in this study were identical to those used by Bernsohn (12).

The flasks contained 0.1 ml. 0.02 *M* ATP; 0.1 ml. 0.27 *M* $MgCl_2$; 0.3 ml. 0.23 *M* glucose; 0.3 ml. 10^{-6} *M* cyctochrome C; 0.3 ml. 0.23 *M* KF; 0.3 ml. 0.03 *M* substrate; and 0.3 ml. homogenate. In those experiments where drugs were added to the flasks, 0.15 ml. 0.01 *M* chlorpromazine; 0.3 ml. 0.01 *M* 5-HT; or 0.3 ml. 10^{-3} *M* to 10^{-6} *M* LSD was added. The volume of fluid in all flasks was then adjusted to 3.0 ml. with 0.05 *M* phosphate buffer. In the experiments where endogenous respiration was determined, the fumarate-pyruvate mixture was replaced by buffer.

Oxygen consumption was measured by the direct method of Warburg. The temperature of the bath was 37°, the gas phase was air, and the equilibration period was ten minutes. At least eight animals were used for each experiment and all measurements were performed in triplicate.

To some of the animals chlorpromazine, 25 mg./Kg., was administered intraperitoneally. An hour after the administration of the drug these animals were sacrificed and the brains homogenized as outlined above.

In the experiments where the brains were used for determination of 5-HT as well as for measurement of oxygen uptake, two animals were sacrificed. Both brains were homogenized together in two volumes of cold distilled water. Equal volumes of this homogenate and buffer were mixed and used as before for the measurement of oxygen uptake. Enough homogenate was mixed with buffer so that at least six different flasks could be run. The rats used in alternate experiments were injected with chlorpromazine, 25 mg./Kg., intraperitoneally. The other runs were considered to be controls.

For the determination of 5-HT the method of Udenfriend, *et al.* (14), was used, samples being analyzed with the Aminco-Bowman Spectrofluorophotometer. Four and one-half milliliters of homogenate were used for each determination.

RESULTS AND DISCUSSION

All experimental results were subjected to statistical analysis and the standard error, E, determined from the equation $E = \sqrt{\Sigma d^2/[n(n-1)]}$. Results were considered to be significant if $m_1 - m_2 > 2\sqrt{E_1^2 + E_2^2}$. The results are reported in Table I.

TABLE I

Drug	$Mm.^3 O_2$ Uptake/50 mg. Tissue/60 min.	Inhibition, %
None	171 ± 6	..
Chlorpromazine (5×10^{-4} *M*)	15 ± 1	91
Chlorpromazine (5×10^{-4} *M*) plus 5-HT (10^{-3} *M*)	33 ± 2	81
5-HT (10^{-3} *M*)	144 ± 3	16
LSD (10^{-4} *M*)	154 ± 8	10
Chlorpromazine (5×10^{-4} *M*) plus LSD (10^{-4} *M*)	13 ± 1	92
5-HT (10^{-3} *M*) plus LSD (10^{-4} *M*)	147 ± 4	14
Chlorpromazine (25 mg./Kg. injected intraperitoneally)	166 ± 7	3

It will be seen from Table I that chlorpromazine, at the stated concentration, produced approximately 90% inhibition of respiration, *in vitro*. This result confirms the finding of Bernsohn (12), who suggested that the inhibition of oxygen uptake is produced through interference, by chlorpromazine, with the hydrogen transport system. When chlorpromazine, 25 mg./Kg., was administered intraperitoneally to rats the oxygen uptake of homogenates prepared from the brains of these animals was not significantly different from that

obtained with homogenates prepared from brains of animals to which the drug had not been administered. The dose of chlorpromazine used was one-third of the reported 14-day LD_{50} (15) and was chosen because it was felt that such a high dose would be sufficient to demonstrate any effects on respiration due to chlorpromazine. The failure of the drug, at this dosage level, to exhibit inhibition of respiration would tend to cast doubt on the concept that the *in vivo* effects of chlorpromazine are due to an interference with the utilization of oxygen. If the assumption is made that the distribution of chlorpromazine in rat brain is similar to the distribution in dogs, as reported by Salzman and Brodie (16), the concentration in rat brain, an hour after the intraperitoneal administration of 25 mg./Kg. would be of the order of 3×10^{-4} M/100 mg. of brain. Bernsohn (12) found that concentrations greater than 10^{-4} M were necessary in order to demonstrate inhibition of oxygen uptake of brain, *in vitro*. The concentration of chlorpromazine necessary to produce inhibition of respiration, *in vitro* is, therefore, in excess of the usual therapeutic dose if a uniform distribution of the drug in the brain is assumed. It has been reported, however, that all of the areas of the brain are not equally susceptible to chlorpromazine (17), and that some areas may be more sensitive to this drug than are other areas.

The addition of 5-HT to the Warburg vessels produced a slight inhibition of oxygen uptake. Although the inhibition produced was found to be significant, it would seem improbable that so slight an inhibition could be considered the primary mechanism whereby 5-HT produces *in vivo* effects. When 5-HT was added to those flasks containing chlorpromazine, a slight reversal of the inhibitory effect of chlorpromazine was observed. It seems of interest to note that the contractile effect elicited by 5-HT on smooth muscle is antagonized by chlorpromazine (18). It has been shown, however, that reserpine appears to cause a release of 5-HT in brain (6) and that reserpine and chloropromazine produce similar, rather than antagonistic, effects on the central nervous system (9).

When LSD was added to the flasks no significant alteration in respiration was observed. This finding would be in agreement with the belief of Bain (19) but in opposition to the results obtained by Mayer-Gross, *et al.* (20), who reported that 10^{-6} M LSD produced a 30% stimulation of respiration, *in vitro*. These results would also tend to be in keeping with the evidence that LSD has no effect upon the duration of narcosis produced by barbiturates (7), especially since the interference with respiratory processes has been suggested as a mode of action of the barbiturates, *in vivo* (13). It was also noted during this study that the addition of LSD to flasks containing either 5-HT or chlorpromazine had no significant effect upon the inhibition of respiration produced by these two drugs. LSD is, however, able to reverse the potentiating action of 5-HT and of chlorpromazine upon the narcosis produced by barbiturates (10).

It was determined that the level of 5-HT in rat brain was approximately 4×10^{-3} μM per Gm. of brain. The previous intraperitoneal injection of chlorpromazine, 25 mg./Kg., produced no appreciable effect on the 5-HT levels. This finding corroborates that of Brodie (8), who found that chlorpromazine does not cause the release of 5-HT in the brain.

SUMMARY

1. The effects of chlorpromazine, 5-HT, and LSD on the oxygen uptake of rat brain homogenate have been studied.

2. Chlorpromazine was found to produce a marked inhibition of oxygen uptake. 5-HT produced a slight inhibitory effect.

3. LSD produced no significant effect upon the respiration of the homogenates.

4. The inhibition elicited by chlorpromazine or by 5-HT was not altered by LSD.

5. Chlorpromazine, when administered intraperitoneally, elicited no significant effect on respiration of the homogenates.

6. Chlorpromazine, when administered intraperitoneally, did not alter the concentration of 5-HT in the brain.

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Convulsant Properties of Oral Pentylenetetrazol Mixtures*

By EUGENE A. CONRAD†, J. MAXWELL LITTLE, and GEORGE H. ARMSTRONG

Mice received during a three-day period oral pentylenetetrazol, reserpine, and niacin singly or in combination prior to challenge with oral pentylenetetrazol. Niacin (20 mg./Kg./day) and pentylenetetrazol (50 mg./Kg./day) did not significantly influence the challenge CD_{50} and TD_{50}. Reserpine (8 mg./Kg./day) induced an apparent lowering of CD_{50} and a significant decrease in TD_{50}; dietary limitation led to similar results. The administration of a mixture containing the above concentrations of pentylenetetrazol and niacin, and reserpine 0.5 mg./Kg./day did not significantly affect the subsequent challenge CD_{50} and TD_{50}.

THOMPSON AND PROCTOR (1) observed a clinical improvement in 46 of 60 elderly psychiatric patients treated with subconvulsive dosages of pentylenetetrazol-niacin mixtures. The potent tranquillizing action of reserpine suggested the addition of this drug might improve the therapeutic effectiveness of the above mixture. Although reserpine possesses ataractic properties, Chen and Bohner (2), and others, reported a lowered threshold for pentylenetetrazol convulsions following premedication with toxic doses of reserpine. For this reason, the present experiments were designed to study the influence of oral reserpine alone, or in combination with pentylenetetrazol and niacin, on the convulsant seizures induced subsequently by challenge with oral pentylenetetrazol.

MATERIALS AND METHODS

Healthy female albino mice weighing 18–25 Gm. were used throughout this study. The animals were fed *ad libitum* except during the times noted in the dosage schedule.

Pentylenetetrazol and niacin were dissolved in physiological saline solution in such concentrations that 1 cc. would be administered by stomach tube per 25 Gm. of animal weight. Reserpine or it's combinations were prepared similarly by suspension in 0.5% aqueous methylcellulose solution using a glass Potter-Elvehjem homogenizer.

During the three-day premedication period the mice were fasted each day for three hours (9 a. m.–noon) and then given, orally, one-half the daily dose of reserpine, or pentylenetetrazol, or a combination of both with niacin; the fast was continued until 4 p. m. when the oral administration was repeated and the animals allowed to eat *ad libitum*. Such regimens led to a significant change in final body weight; therefore, the influence of dietary restriction alone was also investigated. One group of mice was permitted access to a limited amount of food in order to lead to a weight loss of 15–20% during a three-day period.

On the fourth day of the experimental period, the mice were fasted for three hours and then challenged with varying doses of oral pentylenetetrazol; a minimum of 10 mice were employed for each dosage. All mice were observed for thirty minutes following challenge. The numbers of animals showing clonic and tonic convulsions were noted and the results analyzed by the method of Litchfield and Wilcoxon (3).

RESULTS

Premedication with oral pentylenetetrazol, niacin, or reserpine led to significant changes in final body weight as noted in Table I. The subacute administration of a subthreshold dose of pentylenetetrazol induced a gain while the other regimens resulted in a loss in body weight.

Previous treatment with subconvulsive amounts of pentylenetetrazol or niacin did not influence significantly the CD_{50} or TD_{50} of pentylenetetrazol challenge. Reserpine (0.5 mg./Kg./day) administered alone induced a lowering of TD_{50} (borderline significance); however, the mixture containing this amount of reserpine did not significantly influence the TD_{50}. The higher dose of reserpine (8 mg./Kg./day) led to an apparent lowering of CD_{50} and significant decrease in TD_{50}.

The limitation of daily dietary intake resulted in a significantly lowered threshold for tonic convulsions and an apparent decrease in CD_{50}.

DISCUSSION

The data presented confirm the previous observation of a lowered threshold for pentylenetetrazol tonic seizures by subacute premedication with toxic dosages of reserpine. Jenney and Pfeiffer (4) reported a 46% decrease in pentylenetetrazol threshold following dosing with reserpine (50 mg./Kg./day) for nine days; in the present study, premedication with 8 mg./Kg./day for three days led to a 34% diminution in TD_{50}. This alteration in threshold may be due, in part, to the accompanying metabolic changes (as reflected in body weight loss) resulting from reserpine toxicity; the percentage lowering of TD_{50} noted following dietary limitation closely resembles that noted after premedication with reserpine 8 mg./Kg./day.

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The authors are indebted to Mrs. Lulu H. Patrick for the statistical analyses.

TABLE I.—THE INFLUENCE OF SUBACUTE PREMEDICATION ON CONVULSIONS INDUCED BY ORAL PENTYLENETETRAZOL

Daily Regimen	Change in Body Weight, %	Clonic Dose[50]	Tonic Dose[50]
Control (131)[a]	+0.7(±0.8)[b]	89(78–101)[c]	134(120–149)
P-50[d] (40)	+5.4(±1.1) P<0.01	108(92–127) P>0.05	142(123–163) P>0.05
N-20[e] (47)	−4.2(±1.0) P<0.01	85(70–104) P>0.05	134(112–160) P>0.05
R-0.5[f] (47)	−6.1(±0.9) P<0.01	85(75–96) P>0.05	113(101–126) P = 0.05
R-8 (40)	−10.6(±1.3) P<0.01	66(51–86) P = 0.05	88(76–103) P<0.05
P, N, R-0.5 (75)	−4.3(±0.7) P<0.01	78(64–95) P>0.05	125(111–141) P>0.05
Diet limitation (49)	−19.4(±0.8) P<0.01	69(60–80) P<0.05	83(70–99) P<0.05

[a] Number of animals tested.

[b] Mean values ± S. E. as computed by S. D. = $\sqrt{\frac{\Sigma(x - m)^2}{N}}$ and S. E. = $\frac{\text{S. D.}}{\sqrt{N}}$ probability values determined from Fisher's table of t.

[c] mg./Kg. body weight; confidence limits for 19/20 cases in parentheses.

[d] Pentylenetetrazol (50 mg./Kg./day).

[e] Niacin (20 mg./Kg./day).

[f] Reserpine (0.5 mg./Kg./day).

Premedication with reserpine (0.5 mg./Kg./day) alone or admixed with niacin and pentylenetetrazol failed to induce a highly significant lowering of CD_{50} or TD_{50}. This concentration of reserpine represents an amount greater than the maximal chronic oral tolerated doses noted in mice by Schneider (5); the latter was based on changes in growth rate and survival time (6). Also, it has been observed in clinical trials that chronic administration of an oral mixture of pentylenetetrazol, niacin, and reserpine to senile psychotics did not precipitate convulsive seizures (7).

An apparent lowering of CD_{50} was noted in mice receiving a limited diet or reserpine 8 mg./Kg./day. Many of these animals showed, initially, several clonic jerks followed almost immediately by a full tonic extension of the hind limbs. However, Chen and Bohner (2) did not observe a potentiation of clonic seizures in mice premedicated with reserpine (8 mg./Kg. intraperitoneally) and challenged with intravenous pentylenetetrazol.

SUMMARY

Subacute premedication with an oral mixture containing pentylenetetrazol, niacin, and a therapeutic dose of reserpine did not significantly influence the CD_{50} or TD_{50} of subsequent challenge with oral pentylenetetrazol. A limitation of dietary intake sufficient to induce a loss of 15–20 per cent in final body weight induced a significant lowering of pentylenetetrazol TD_{50}.

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A Note on the Alkaloids of *Vinca rosea* Linn. (*Catharanthus roseus* G. Don.) II*

Catharanthine, Lochnericine, Vindolinine, and Vindoline

By MARVIN GORMAN, NORBERT NEUSS, GORDON H. SVOBODA, ALBERT J. BARNES, Jr., and NANCY J. CONE

VARIOUS INVESTIGATORS have isolated from *Vinca rosea* Linn. the known alkaloids ajmalicine (1–4), akuammine (2), tetrahydroalstonine (1, 4), serpentine (3, 4), lochnerine (1–3), and reserpine (5). In addition to these the new alkaloids, vindoline (6) and vincaleucoblastine (7), have been reported. In an earlier communication one of us has confirmed the presence of tetrahydroalstonine, ajmalicine, and lochnerine and reported the isolation of three new compounds leurosine, perivine, and virosine (1).

Since that time we have been able to isolate from this plant[1] three additional, apparently new alkaloids: catharanthine, lochnericine, and vindolinine, as well as confirming the presence of vincaleucoblastine, serpentine, and vindoline.

The present note describes the characterization of these three new alkaloids as well as the partial structure of vindoline.

Chromatography of a larger batch (1) of the weakly basic fraction yielded the following alkaloids in the order of their elution: catharanthine, vindolinine, tetrahydroalstonine, ajmalicine, vindoline, leurosine, vincaleucoblastine, and virosine.[2] Lochnericine is obtained by the chromatography of an alkaloidal fraction prepared from a hexane extract of the plant. Serpentine was isolated as its nitrate by chromatography of the strong bases obtained from an alkaline benzene extract (1).

Catharanthine crystallizes from methanol, m. p. 126–128°, $[\alpha]_D^{27} = +29.8°$ ($CHCl_3$), pK'a = 6.8 (66% DMF), and is a $C_{21}H_{24}O_2N_2$ compound. It forms different solvates depending upon conditions accompanying crystallization and drying.

Anal.—Calcd. for $C_{21}H_{24}O_2N_2 \cdot H_2O$: C, 71.16; H, 7.39; N, 7.90. Found: C, 71.02; H, 7.38; N, 7.71.

Its sulfate recrystallized from alcohol, m. p. 164–167° (decompn.).

Anal.—Calcd. for $C_{21}H_{24}O_2N_2 \cdot {}^1/_2H_2SO_4 \cdot {}^1/_2H_2O$: C, 63.94; H, 6.64; N, 7.10; S, 4.06. Found: C, 64.03; H, 6.76; N, 6.91; S, 3.90.

The two oxygen atoms of catharanthine are contained in a carbomethoxyl moiety as evidenced by the reduction with lithium aluminum hydride to give catharanthinol, m. p. 227–228° (decompn.) from methanol.

Anal.—Calcd. for $C_{20}H_{24}ON_2$: C, 77.88; H, 7.84; N, 9.08; mol. wt., 308.4. Found: C, 77.73; H, 7.85; N, 8.85; mol. wt., 309.3 (X-ray data).

The base readily forms a methiodide with excess methyl iodide in benzene. Crystallization from methanol yields material m. p. 232–234° (decompn.).

Anal.—Calcd. for $C_{21}H_{24}O_2N_2 \cdot CH_3I$: C, 55.23; H, 5.69; N, 5.86; I, 26.53. Found: C, 55.07; H, 5.63; N, 5.89; I, 26.71.

The ultraviolet spectrum of catharanthine is typical of an unsubstituted (8) indole alkaloid: $\lambda_{max.}^{EtOH}$ 226 mμ (log a_M 4.56), 284 mμ (log a_M 3.92), 292 mμ (log a_M 3.88); $\lambda_{min.}^{EtOH}$ 250 mμ (log a_M 3.39).

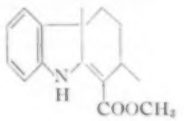
Its infrared spectrum has the following characteristic bands: $\lambda_{max.}^{CHCl_3}$ 2.92, 5.81, 7.92, 9.29 μ. Catharanthine readily absorbs one mole of hydrogen with PtO_2 catalyst in alcohol, indicating an isolated double bond. Thus the empirical formula of this alkaloid allows the assignment of its nucleus to a pentacyclic fused ring system with the partial formula I.

$$C_{19}H_{21}N_2(COOCH_3)$$

I

Lochnericine crystallized from methanol, m. p. 190–193° (decompn.), $[\alpha]_D^{27} = -432°$ ($CHCl_3$), pK'a = 4.2 (66% DMF).

Anal.—Calcd. for $C_{21}H_{24}O_3N_2$: C, 71.57: H, 6.86; N, 7.95; mol. wt., 352.4; (1) OCH_3, 8.86. Found: C, 71.49; 71.43: H, 6.97; 6.71; N, 8.10; mol. wt., 352 (X-ray data); (1) OCH_3, 9.10.



II

The spectral properties of lochnericine, as well as the high negative value for its rotation, indicate that this alkaloid contains the same type of chromophore (II) as tabersonine (9), akuammicine (10), and echitamidine (9).

The ultraviolet spectrum is characterized by the following absorption: $\lambda_{max.}^{EtOH}$ 226 mμ (log a_M 4.00), 297 mμ (log a_M 4.04), 327 mμ (log a_M 4.23); $\lambda_{min.}^{EtOH}$ 215 mμ (log a_M 3.90), 256 mμ (log a_M 2.86), 305 mμ (log a_M 4.03).

The infrared spectrum in $CHCl_3$ solution shows the following prominent bands: $\lambda_{max.}^{CHCl_3}$ 2.96, 3.40, 3.59, 5.96, 6.19, 7.97 μ.

Vindolinine could not be induced to crystallize as a free base. Its dihydrochloride crystallizes readily from methanol-ether, m. p. 210–212 (decompn.), $[\alpha]_D^{27} = -8°$ (H_2O), pK'a = 3.3, 7.1 (66% DMF).

* Received March 17, 1959, from Lilly Research Laboratories and Organic Chemical Development, Eli Lilly and Co., Indianapolis, Ind.

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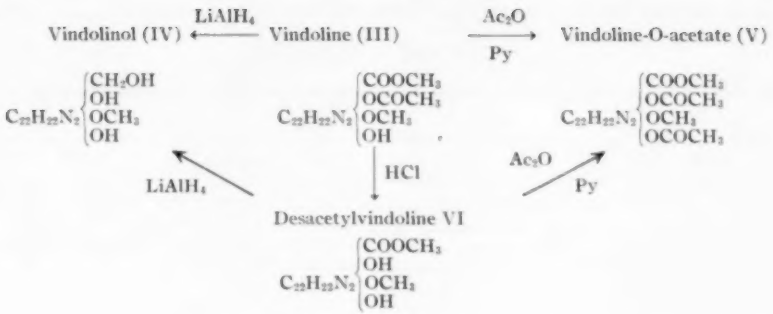
[1] The drug used in this study was obtained from Meer Corp. and was botanically authenticated by Dr. V. H. Madis.

[2] All known alkaloids were identified by comparison of melting points, X-ray powder diffraction patterns, infrared and ultraviolet spectra with those of the authentic specimen.



The ultraviolet spectrum is typical of a dihydroindole alkaloid (11): $\lambda_{max.}^{EtOH}$ 245 mμ (log a_M 3.83), 300 mμ (log a_M 3.37); $\lambda_{min.}^{EtOH}$ 271 mμ (log a_M 2.98).

The infrared spectrum shows the following characteristic bands: $\lambda_{max.}^{Nujol}$ 5.77, 6.43, 8.21 μ.

Anal.—Calcd. for $C_{23}H_{24-26}O_2N_2 \cdot 2HCl$: C, 61.61; 61.31; H, 6.40; 6.86; N, 6.84; 6.81; Cl, 17.32; 17.24. Found: C, 61.54; 61.44; H, 6.56; 6.58; N, 6.58; 6.64; Cl, 17.03.

It forms a picrate which, when recrystallized from acetone-water, melts at 268–272° (decompn.).

Anal.—Calcd. for $C_{23}H_{24-26}O_2N_2 \cdot C_6H_3O_7N_3$: C, 57.34; 57.14; H, 4.81; 5.15; N, 12.38; 12.34. Found: C, 57.01; H, 4.77; N, 12.31.

Vindoline crystallizes from ether, m. p. 154–155°, $[\alpha]_D^{27}$ = +42° ($CHCl_3$), pK'a = 5.5 (66% DMF), and is a $C_{25}H_{32}O_6N_2$ compound.

Anal.—Calcd. for $C_{25}H_{32}O_6N_2$: C, 65.77; H, 7.07; N, 6.14. Found: C, 65.99; H, 7.02; N, 6.04.

Its hydrochloride, recrystallized from acetone, melts at 161–164°.

Anal.—Calcd. for $C_{25}H_{32}O_6N_2 \cdot HCl$: C, 60.90; H, 6.75; N, 5.68; Cl, 7.19. Found: C, 61.17; H, 6.83; N, 5.77; Cl, 6.99.

The ultraviolet absorption spectrum of the alkaloid suggests a dihydroindole moiety (11): $\lambda_{max.}^{EtOH}$ 212 mμ (log a_M 4.49), 250 mμ (log a_M 3.74), 304 mμ (log a_M 3.57); $\lambda_{min.}^{EtOH}$ 241 mμ (log a_M 3.71), 274 mμ (log a_M 3.02).

Its infrared spectrum contains the following characteristic bands: $\lambda_{max.}^{CHCl_3}$ 3.37, 3.42, 3.51, 3.56, 3.7–4.1 (shoulder), 5.74, 6.20, 8.1, 9.7, 12.0, 12.2 μ.

Vindoline readily forms an O-acetate with pyridine in Ac_2O, m. p. 127–128.5°[2]; disappearance of shoulder 3.7–4.1 in the infrared spectrum.

Anal.—Calcd. for $C_{27}H_{34}O_7N_2$: C, 65.04; H, 6.87; N, 5.62; CH_3CO (2), 17.27. Found: C, 65.03; H, 6.99; N, 5.43; CH_3CO (2), 17.45.

Lithium aluminum hydride reduction in a mixture of ether and tetrahydrofuran yields the corresponding alcohol, vindolinol.

The infrared spectrum showed no carbonyl absorption and two new hydroxyl bands were present: $\lambda_{max.}^{CHCl_3}$ 2.84, 2.95 μ, m. p. 172–176°.[2]

Anal.—Calcd. for $C_{23}H_{30}O_6N_2$: C, 68.34; H, 7.82; N, 7.82. Found: C, 68.46; H, 7.85; N, 7.40.

Treatment of vindoline with concentrated hydrochloric acid (eight minutes, reflux) removes the acetyl group with formation of desacetylvindoline, m. p. 156–157°[2], $\lambda_{max.}^{CHCl_3}$ 2.84 μ.

Anal.—Calcd. for $C_{24}H_{30}O_6N_2$: C, 66.64; H, 7.30; N, 6.76. Found: C, 66.61; H, 7.37; N, 6.83.

Reduction of desacetylvindoline with lithium aluminum hydride yields vindolinol, while acetylation with acetic anhydride in pyridine gives vindoline-O-acetate.

Vindoline readily absorbs one mole of hydrogen with PtO_2 in alcohol indicating an isolated double bond.

Simultaneous determination of O-CH_3 and (N)-CH_3 (Zeisel) on vindoline and vindolinol gave the following results:

Vindoline; *Anal.*—Calcd. for N-CH_3 (1), 3.29; O-CH_3 (2), 13.6. Found: N-CH_3, 1.29; O-CH_3, 18.29.

Vindolinol; *Anal.*—Calcd. for N-CH_3 (1), 3.89; O-CH_3 (1), 8.03. Found: N-CH_3, 3.53; O-CH_3, 11.29.

Since the conversion of N- to O-methyl is known (12), the results above indicate the presence of one N-CH_3 group and one O-CH_3 ether in vindoline and its derivatives.

Analyses of vindoline, its salts, and transformation products allow establishing of partial structures for these compounds (III–VI) and clearly indicate that vindoline is a $C_{25}H_{32}O_6N_2$ compound containing a fused five-ring system, rather than a $C_{27}H_{34}O_7N_2$ as postulated by Kamat, *et al.* (6).

The work toward the structure elucidation of these alkaloids, now in progress in these laboratories, will be published later.

[2] Ultraviolet spectrum like that of vindoline.

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Book Notices

Scale-Up in Practice. Edited by RICHARD FLEMING. Reinhold Publishing Corp., New York, 1958. iv + 134 pp. 12.5 x 18.5 cm. Price $4.50.

Pilot plant problems are discussed in this book. Chapters are included on the factors justifying the operation of pilot plants; a brief review of scale-up theory; simulating operation of processes and equipment with computers; the hazards involved in scale-up; the process and business economics of scale-up; and organizing for scale-up.

Psychopharmacology Frontiers. Edited by NATHAN S. KLINE. Little, Brown and Co., Boston, 1959. xxiii + 533. 15.5 x 23.5 cm. Price $10.

This book is composed of a series of papers by experts in their respective fields which were presented to a Psychopharmacology Symposium of the Second International Congress of Psychiatry, held in Zurich, Switzerland, on September 2–4, 1957. This volume should serve as a reference book and as a working guide to the compex field of psychopharmacology.

Psychotherapeutic Drugs. By ASHTON L. WELSH. Charles C Thomas, Springfield, 1958. xiii + 139 pp. 15 x 22.5 cm. Price $4.75.

This monograph is designed to describe the various psychotherapeutic agents, their indications for use, and their side effects. The book is divided into five parts, each part devoted to a discussion of one of the major groups of tranquilizers including phenothiazines, rauwolfia alkaloids, substituted propanediols, diphenylmethane derivatives, and ureides and amides.

The Evaluation of Drug Toxicity. A Symposium. Edited by A. L. WALPOLE and A. SPINKS. Little, Brown and Co., Boston, 1958. xi + 138 pp. 15.5 x 23.5 cm.

Included in this book is a group of papers presented to a symposium held in October 1957 in observance of the dedication of the new research laboratories of Imperial Chemical Industries (Pharmaceutical Division). Many phases of the evaluation of drug toxicity are discussed.

Acetophenetidin. By PAUL K. SMITH. Interscience Publishers, New York, 1958. x + 180 pp. 15.5 x 23.5 cm. Price 5.75.

This monograph is the fourth in a series of critical reviews of the literature on analgesic and sedative drugs. The first volume dealt with acetanilid, the second with salicylates, and the third with antipyrine. In the monograph on acetophenetidin, a critical bibliographic review is presented which makes available all of the pertinent literature in a convenient form. It should serve as an aid to future investigators by calling their attention to the inadequacies of past work so that these may be a guide to planning more definitive experiments. Acetaminophen (*N*-acetyl-*p*-aminophenol), the major metabolite of acetophenetidin is believed to be responsible for the therapeutic activity of acetophenetidin. Because of the close relationship existing between these two drugs, the author has included in this volume many references to the increasing number of studies dealing with *N*-acetyl-*p*-aminophenol.

Advances in Pest Control Research. Vol. II. Edited by R. L. METCALF. Interscience Publishers, New York, 1958. vii + 426 pp. 15 x 2 cm. Price $12.50.

This is the second in a series of selected contributions by specialists in fields related to pest control research. The first volume of the series was reviewed in THIS JOURNAL, **47,** 230(1958). The scope of Volume II is indicated by the chapter titles which are: The fluid kinetics of application of pesticidal chemicals; Innate toxicity of fungicides; Isotope dilution techniques for the determination of pesticide residues; Wool digestion and mothproofing; The relation of chemical structure to activity for the 2,4-D-type herbicide and plant growth regulator; Chemical structure and activity of DDT analogues with special consideration of their spatial structures; and The spread of insecticide resistance in pest species.

Bacteriophages. By MARK H. ADAMS. Interscience Publishers, New York, 1959. xviii + 592 pp. 15 x 23 cm. Price $15.

Phage research has had an erratic history, and this is probably the first comprehensive book on bacteriophages. It should be useful to every student of modern biology.

Reversible Renal Insufficiency. Diagnosis and Treatment. By DONALD H. ATLAS and PETER GABERMAN. The William and Wilkins Co., Baltimore, 1958. ix + 233 pp. 14.5 x 21.5 cm. Price $7.

This monograph on reversible acute and chronic renal insufficiency is intended to be of particular value to nephrologists, physiologists, and internists, as well as to general practitioners and medical students. It is a comprehensive review of the specific field.

Year Book of Drug Therapy. 1958–59 Series. Edited by HARRY BECKMAN. The Year Book Publishers, Chicago, 1959. 569 pp. 13 x 19.5 cm. Price $7.50.

This volume represents Dr. Beckman's review of medical literature for the "series year" beginning September 1958. His concise, informative reporting is up to his established high standards. Occasional editorial comments, such as his criticism of poor trial design for clinical testing of new neuropsychiatric tranquilizers and stimulants, are interesting. The series is a useful reflection of therapeutic progress, and is a valuable reference collection. Good indexes are appended.

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